EXHIBIT 27

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Please find below a communication from the EXAMINER in charge of this application.

Commissioner of Patents

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BEFORE THE BOARD OF PATENT
APPEALS AND INTERFERENCES

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Application Serial Number: 08/252,384
Filing Date: 1 June 1994
Appellant(s): McDaniel *et al.*

C. Steven McDaniel
For Appellant

EXAMINER'S ANSWER

This is in response to appellant's brief on appeal filed 16 August 1995
and the supplemental appeal brief filed 24 November 1995

(1) Real Party of Interest.

A statement identifying the real party in interest is contained in the brief.

(2) Related appeals and interferences.

A statement identifying the related appeals and interferences which will directly affect or be directly affected by or have a bearing on the decision in the pending appeal is contained in the brief.

(3) Status of the claims.

The statement of the status of the claims contained in the brief is correct.

(4) Status of the Amendments after final.

The appellant's statement of the status of amendments after final rejection contained in the brief is correct.

(5) Summary of the invention.

The summary of the invention contained in the brief is noted but is not a "concise" explanation of the invention. The invention is defined by the claims on appeal (of which the claims fall together).

Insofar as the summary presented in the brief starts at page 5 and ends at page 10, it is not a concise summary. The commentary regarding Japanese terrorists and Saddam Hussein is noted but is not germane to the claimed invention nor is any part of that discussion supported by direct recitation in the present application written description and claims on appeal. All of these events occurred after the effective filing date of the present application.

The commentary in the paragraph bridging pages 5-6 discusses treatment of toxic compounds in connection with illness(es), however, neither treatment of illness(es) nor the effects of toxic compounds are the presently claimed invention. Thus, the discussion of the footnote at page 5 is not directed to the presently claimed invention.

The discussion of U.S. Department of Defense experience is also not supported by direct recitation in the present application, and therefore, cannot be relied upon as part of the summary. Page 6, first full paragraph asserts long felt and unfulfilled need, but it is not apparent from the instant application written description as filed which contains no disclosure of U.S. Department of Defense experience. Here, "long felt need" (*Texas Instruments Inc. v. International Trade Commission*, 26 USPQ2d 1018 (Fed. Cir. 1993)) is analyzed as of the date of the articulated identified problem and evidence of efforts to solve that problem. The present appeal brief summary (pages 5-10) sets a date of "long felt need" by discussion of Japanese subway terrorists and Saddam Hussein's threat to use nerve agents in Operation Desert Storm and its use against Kurdish rebels. None of these events occurred prior to the effective filing date of the present application nor do any of these events appear to directly refer to the subject matter defined by the present claims. Furthermore, each of the foregoing events occurred after the effective filing date of the present application filing, and thus, do not show a long felt need because they do not demonstrate a date that is prior to the instant application effective filing date. Dates later than the instant application filing date do not demonstrate nor provide any nexus to any "long filed need".

Page 6 (first full paragraph) of the appeal brief asserts that the present invention provides an alternative and alleges that short of the commercial incentive of patent protection it is not likely that alternatives would be commercialized. This is not persuasive because of (1) the absence of factual scientific evidence in the application as filed that substantiates the assertion of short of the commercial incentive of patent protection it is not likely that alternatives would be commercialized, (2) there is no nexus demonstrated in the present application as originally filed of commercialization nor is funding for research considered commercialization, (3) there is no demonstration of long-felt need, and (4) commercialization is not the presently claimed invention.

Page 6 second full paragraph of the instant brief asserts that

> "Prior to the present invention, it was known, that microorganisms could degrade organophosphorous compounds used as pesticides. It was also known that certain bacteria exhibited a particular penchant for doing so. At least certain bacteria appeared to be able to attack a wide array of OPs."

which is a statement that the processes were already known, and were known to use the microorganisms that contained the protein that acted on and detoxified organophosphorous compounds. Thus, the presently claimed method of use of the protein was already known.

Insofar as "recombinant" is recited, Watson *et al.* indicate that all DNA is recombinant which makes the protein made from the template of that DNA also a recombinant protein and is not distinguished by the present claims. Thus, the comments in the second full paragraph of page 6 are not persuasive that the presently claimed invention set forth in the present claims on appeal are distinguished. They are not. This paragraph also discusses whether or not the reaction was enzymatic. Such comments are not persuasive because the present claims only "expose" the organophosphorous compound. Exposing is not an explicitly recited step of enzymatic reaction.

It is also noted that this paragraph makes the one sentence remark as to whether or not there was one or a multitude of enzymes, however, the presently claimed invention defined by the appealed claims does not distinguish between these as no claim indicates that the organophosphorous acid anhydrase is isolated and purified as a single protein or used as such. This paragraph also asserts that it was not known whether or not the protein would breakdown the more recalcitrant organophosphorous compounds such as those with P-S linkages as found in certain nerve gases. This also not persuasive in the summary since no appealed claims defining the presently claimed invention refer to nor even *per se* recite recalcitrant organophosphorous compounds such as those with P-S linkages. Thus, the comments in the summary of appellant's brief are not persuasive in regard to the presently claimed invention.

The summary in the appeal brief also asserts (paragraph bridging pages 6-7) that there are roadblocks to overcome such as the sequencing of the DNA and various assays that are referred to in the present specification at page 4 line 23 to page 5 line 5. These pages of the present specification, however, discuss the cloning of the gene (the present claims are not directed to the cloning of the gene but to a method of use of the enzyme which is a protein. The protein is not a DNA.

As to assertion of commercial basis, the paragraph discusses expression of the gene. Gene expression is not the presently claimed method of use of the protein. See the claims on appeal. As far as this paragraph discusses attempts to characterize the enzyme, the present claims are not directed to a method of characterization of the enzyme but to a method of use of the enzyme, where from the cited prior art, it is known that the enzyme catalyzes a reaction that detoxifies, i.e., degrades organophosphorous compounds. The appeal brief at page 6, last full paragraph admits that the art contained knowledge of the presence of the enzyme and that the enzyme catalyzed degradation of organophosphorous compounds. The present application also discloses in the last paragraph of page 7 that the DNA was cloned, expressed, and the results published in peer reviewed journals prior to filing date of the present application. Thus, the comments in the paragraph bridging pages 6-7 are not persuasive of appellant's comments in the brief.

The first and second full paragraphs of page 7 of the present appeal brief assert that it was not known where in the bacterial genome the gene was located nor whether the enzyme could be expressed in another cell other than the one in which they originated. These comments are not persuasive in view of the cited references of McDaniel *et al.*(BY), Harper *et al.* (BX), Wild *et al.* (AT), McDaniel (AZ), and for example, Mulbry *et al.* (J. Bacteriol., 1989). Each reference indicates that prior to the time the present invention was made that a gene for the enzyme was known and cloned into a host cell other than the one in which the DNA encoding the enzyme originated. Note also that the McDaniel (AZ) reference is contrary to the statements made in the present appeal brief (by McDaniel, for appellant, one of whom is McDaniel) in the paragraph bridging pages 6-7 indicates in the abstract (pages iv-v) that "... a useful plate assay for the detection of Opd$^+$ colonies was developed and used ...". Note the approval by six (6) others (i.e., the McDaniel dissertation committee). Thus, from the references indicated above, it is apparent that the gene encoding the enzyme that degraded organophosphorous compounds had been isolated, cloned, and expressed in heterologous host organisms regardless of whether or not the DNA or the protein had been sequenced (sequencing does not change the physical entity of the DNA nor of the protein but only adds information). Therefore, the present appeal brief assertion that it was not known where in the bacterial genome the gene was located nor whether the enzyme could be expressed in another cell other than the one in which they originated is erroneous and not persuasive.

It is also asserted in the second full paragraph of page 7 that it was not known if the enzyme would have attacked the organophosphorous agents, however, this is not persuasive in view of the above indicated references as McDaniel (BY) at page 2310 indicates that a wide range of pesticides are degraded; Harper *et al.* (BX) at page 2586, for example, indicates that soman (among other organophosphorous compounds is degraded, note the indication in the McDaniel (AZ) dissertation that states that the gene was cloned and refers to citation references 8 (McDaniel *et al.* 1988 J. Bacteriol., 170: 2306-2311), 10 (Mulbry *et al.* 1986 Appl. Environ Microbiol. 51: 926-930), and 12 (Serdar *et al.* 1985 Bio/Technol. 3: 567-571) referred to at page 2586 of the Harper *et al.* reference); Wild *et al.* (AT) indicates that the enzyme (see pages 632 (cloning) and 633 (characterization) degrades organophosphates (Table 1); Mulbry *et al.* (see at least pages 6740) as to the detoxification of organophosphorous waste; and, see for example pages 48-136 of the McDaniel (AZ) reference. Thus, the comments in the comments in the second full paragraph of page 7 are not persuasive.

In the third full paragraph of page 7, the present appeal brief asserts that standard laboratory procedures were unsuccessful (referring to page 4, lines 32-34 of the present application specification). This is not persuasive since the above specification page 4, lines 32-34 indicates that isolation of the plasmids was difficult and difficult is not the same as unsuccessful and is apparently contradictory to the references cited above. Moreover, the cited references are successful examples of cloning the gene which is the antithesis of appellant's assertions for which appellant's are in part authors of the references. Appellant's own references are indicative of the error in appellant's assertions. Thus, the comments in this paragraph of the brief are contrary to the cited references and not supported by the cited page 4, lines 32-34 of the present specification. It is also noted that the third full paragraph asserts failure to achieve acceptable levels of expression. This is not persuasive because the present claims are not directed to a method of/for producing the enzyme *per se* but of a method of use of the enzyme *per se* regardless of the amount (none is recited in the claims) or purity (no claim recites a process where the enzyme is purified) where the present claims do not indicate any specific levels of expression of the DNA. The present method as claimed is not to a process of gene expression, it is to a process of using an enzyme, regardless of how it is or was produced. The method of use of the enzyme is what is claimed. It is a different statutory class of invention from that of

making the enzyme which is argued in part in the brief. Note for example that Harper *et al.* (page 2586) indicates that purified enzyme preparations of the cloned gene product were shown to specifically degrade organophosphorous compounds, i.e., the presently claimed method of use. Here, Mulbry *et al.* indicate (see for example the abstract) that the protein produced from the cloned gene had higher levels of organophosphorous activity. Thus, here, the assertion of inability to achieve acceptable levels of expression is not persuasive as the instant claims do not require acceptable levels of expression and the disclosure in the cited art of purified enzyme preparations of the cloned gene product demonstrates that the ordinary skilled in the art knew of and had obtained acceptable levels of expression that resulted in purified enzyme preparations. For these reasons, the comments in the third full paragraph of the present brief are not persuasive.

In the paragraph bridging pages 7-8, the present appeal brief asserts that accurate sequencing of the gene was a roadblock due to the high G-C to A-T ratio. This is not persuasive because the present claims are not directed to a DNA *per se* and the above indicated references disclose the cloning of the gene encoding the enzyme. Thus, the comments are not persuasive nor correlated to the present claims on appeal that are directed to a method of use of the enzyme (i.e., it is a protein and a protein is not a DNA). As the above references clearly demonstrate, it is not necessary to have sequenced the DNA nor the enzyme for one to have used same.

The first paragraph of page 8 of the present brief refers to difficulty in producing the enzyme for purification and characterization by reference to page 5, lines 1-3 and 5-7 and accurately designing "hook-ups" (note that hook-ups are not disclosed, defined, nor even *per se* discussed in the present application and is jargon) at page 8, lines 28-32 of the present specification. It is pointed out that here, the above asserted difficulty is not persuasive as the Wild *et al.* (AT) reference among other references indicated cloning the DNA encoding the enzyme (see pages 632) and characterizing the protein which was demonstrated to degrade organophosphates (Table 1). It is also pointed out that the discussion of "hook-ups" is also not persuasive nor pertinent to the process of use as the discussion of how the enzyme is obtained does not alter the function of the enzyme in the presently claimed method of use which is what is claimed. The presently claimed method is a "method of use" - see the appendix of claims attached to the appeal and supplemental brief. The discussion in the first full paragraph of

appeal brief page 8 is one of a process of making the enzyme - i.e., it is a distinct and different statutory process from the presently claimed method of use. The minor modifications referred to in this paragraph are not persuasive because the presently claimed invention is not directed to modifications of the enzyme nor to the N-terminus of the enzyme *per se* but to a process of use of the enzyme. Of note is the exhibit B attached to appellant's brief which by appellant's own comparison the originally filed sequence contains missing DNA bases (see sequence 1 of exhibit B compared to sequences 2-6 and sequence 6 is not the originally filed sequence and are not "minor modifications" - as the changes (exhibit B, page 2, first line of bases) inserts an entire codon and would change the sequence from Met-Gln-Thr-Arg-Arg-Val-Val-Leu-Lys-Ser-Ala-Ala-Ala-Gly ... to Met-Gln-Thr-Arg-Arg-Val-Val-Leu-Lys-Ser-Ala-Ala-Ala-Ala-Gly ..., i.e., it adds at least one and more entire amino acids to the sequence at the N-terminal region at the 14th residue and in the remaining part of the enzyme for which the present application makes no explanation for the change. In the second line of bases (page 2 of exhibit B) there is a frame shift of one (1) base that changes the sequence from "... GCG ACG TGG..." to "... GCG AGG GTG..." which changes the encoded amino acids from "... Ala-Thr-Trp ..." into "... Ala-Ser-Val ..." for which all of the amino acids shown in the exhibit change in sequence and/or in identity due to the frame shift. This is "not a minor modification" as the remainder of exhibit B demonstrates at position 182 (third line of bases at page 2 of exhibit B) another reading frame shift of a single base which further changes the DNA. Note the additional modifications that appellant seeks to make as set forth in exhibit B have additional changes that are not supported in the application as originally filed. See the attached marked copy of appellant's exhibit B. Moreover, this paragraph of appellant's brief does not address the process of use of the enzyme as discussion of the problems of making is not what is claimed as the invention. As demonstrated by the references, the isolation and purification of the enzyme did not deter those of ordinary skill in the art from obtaining the enzyme. Thus, appellant's comments are not persuasive.

In the second full paragraph of page 8, the present brief asserts (specification page 8, line 34 to page 10, line 9 and page 10, lines 11-19) that the inventors and others failed to obtain enough enzyme for testing. This is not persuasive because others (see the above cited references) disclosed (1) cloning the gene, (2) the amino acid sequence of the enzyme; (3) testing the enzyme in an assay which is the antithesis of appellant's comments; and, (4) the process of detoxification as claimed is

disclosed in the references. Insofar as the brief asserts that high level expression was elusive, the instant claims do not recite high level expression nor is high level expression needed nor is isolation nor purification of the enzyme needed - note the absence of recitation of same in the claims on appeal. Furthermore, the assertion of high level expression eluding others is not persuasive in view of the present claims on appeal, of which, none recite the asserted high level expression nor the cells with the vectors that effect high level expression. Therefore, the comments in the brief (page 8, second full paragraph) are not persuasive.

The paragraph bridging pages 8-9 of the present brief asserts that once the gene encoding the enzyme was capable of being manipulated a wide variety of uses became possible. So noted, however the wide variety of uses are not what are claimed, rather, the method is directed to detoxifying any organophosphorous compound in the absence of any recitation of an operative recited step of anything that occurs in the method defined by, for example, claim 53. Merely exposing the compound to the organophosphorous acid anhydrase does not necessarily effect or reflect any change to the organophosphorous compound *per se*.

In the first full paragraph of page 9, the brief refers to successes, however, it is not readily apparent as the above exhibit B would appear to demonstrate the failure of the present application written description to set forth the correct sequence else why would there be a necessity to correct same? From the foregoing exhibit B, it is apparent that the present application as originally filed did not (1) set forth the complete and essentially correct DNA sequence nor the correct amino acid sequence nor (2) determine the correct reading frame as is evidenced by the multitude of reading frame changes that appellant refers to in exhibit B attached to the present brief. Moreover, (3) the prior art discloses assays for detecting the enzyme and thus, the commentary regarding the assay for detecting the enzyme are not persuasive. The commentary in the brief of the assay for detecting the enzyme is noted and appellant's indication that the references disclose the presently claimed method of detoxifying organophosphorous compounds. Here, the comment (4) as to expression in bacteria is also noted but not persuasive in view of the references as discussed above and (5) the isolation and purification of the enzyme is not persuasive nor commensurate to the presently claimed invention. Note that the claims do not recite a purified and isolated enzyme and the process of producing the

enzyme in the present claims does not make the protein any different from the enzyme as produced by any other process. Moreover, the present claims are directed to a method of use of the enzyme, not a method of making the enzyme. In item (6), the knowledge gained by the inventors at "designing successful hook-ups" is not what is claimed nor is it described in the claims as such as the present claims are not directed to vectors *per se* nor to a method of constructing same. Thus, the comments in this paragraph of appellant's brief do not summarize the invention.

In the second full paragraph of page 9, appellant asserts that many tried to achieve what appellant's state they have done, however, it is not a novel approach nor is it apparent that appellant unexpectedly or surprisingly found the gene because of the errors apparent in the present application - note the above discussion and the new matter issue. Moreover, from the references discussed above, it is apparent that others (see the cited references) as for example indicated above and in the stated grounds of rejection found the gene. Therefore, it is not unexpected nor surprising. Thus, this paragraph of appellant's brief does not summarize the invention.

In the last full paragraph of page 9, the brief refers to claim 53, and page 13, lines 15 *et seq.* and 20-25 as demonstrative of several methods of using the enzyme, however, this is not persuasive of nor demonstrative of "several methods of using" enzyme as lines 15-36 of page 13 only refer to detoxifying organophosphorous compounds, i.e., it is only one process of use and one (1) is not a multiple. Thus, this paragraph in the brief is not persuasive of nor demonstrates a multiplicity of methods of use.

It is asserted in the first paragraph of page 10 that claim 54 (and refers to page 13, lines 30-34) is a refinement of the method of claim 54. This is nonsensical as a claim is not a refinement of itself. Note that passing the compound through an unspecified matrix as recited in the claim is not a refinement of the method as water is also a matrix of molecules forming the liquid in which the compound and the enzyme are contained. Thus, the comments as to claim 54 are not persuasive. It is also noted that this paragraph discusses claims 55-58 and refers to specification page 13, line 34 to page 14, line 1 6 in reference to filtration and exposure in air or fluid or in gas masks. In each case, the

method is the same as to the effect upon the organophosphorous compound. These are not different methods of use.

The second full paragraph of page 10 refers to claims 59, 60, and 64 and to page 14, lines 18-31 as well as page 14, line 33 to page 15, line 10. Insofar as these claims are asserted in the brief to refer to spraying or introduction into a container or pretreatment with the protein, each relies upon degradation of the organophosphorous compound by the protein (i.e., the same enzymatic process). However, the commentary regarding putting the enzyme in the gut of an animal is not persuasive that the method works. For example, in humans the pH of the stomach is ≤ 1 and contains the strong acid hydrochloric acid which is (1) the wrong pH for the enzyme organophosphorous acid anhydrase (note page 31 of the instant specification that discusses pH 9 as the pH at which enzyme activity is evaluated (i.e., a basic pH as opposed to an acidic pH). Note that here Chaudhry *et al.* disclose that the pH optimum is $7.5 \leq pH \leq 9.5$. Thus, it is apparent that the statement in appellant's brief regarding putting the enzyme in the environment of a human or animal gut would have resulted in the enzyme that was inactive since the gut pH ($pH \leq 1$) is the wrong pH for organophosphorous anhydrase enzymatic action. Moreover it is not readily apparent that the enzyme would have survived the proteolytic degradation by proteases normally found in the gastrointestinal tract of humans or animals. See for example Stryer (page 197) which indicates that trypsin cleaves most peptide bonds following arginine or lysine residues where the present organophosphorous acid anhydrase (see the sequence recited in the claims) contains a plethora of arginine and lysine residues. Thus, the commentary regarding putting the enzyme in the gut of a human or other animal is not persuasive since it is apparent that it would have been degraded by gut enzymes. As to the commentary regarding pretreatment, this is still the same process of the degradation of the organophosphorous compounds. Thus, the comments in the second full paragraph of page 10 are not persuasive of a different method.

The comments in the last full paragraph of page 10 refer to claim 61 and 63, figure 1 of the specification and table 1 (page 9 of the specification) are noted, however, where the comments refer to certain vectors and host cells and transgenic organisms, the claims are not *per se* directed to same but to a method of use of the enzyme. The enzyme is not *per se* the vector nor the gene nor the host cell

nor a transgenic organism. Here, the reference to table 1 is noted, however, materials recited in the table are not *per se* recited in any claim and to read into the claims, limitations from the table is an incorrect reading of the claims - see *In re Zletz*, 13 USPQ 2d 1320 (Fed Cir 1989)

The present claims are not *per se* directed to a DNA, are not *per se* directed to vectors, are not *per se* directed to host cells, and are not *per se* to a process of making the enzyme. The present invention is a method of detoxifying any organophosphorous compound (none are specifically excluded by the present claims on appeal) in the absence of any recitation of any operative recited step of anything that occurs in the method defined by claim 53 as merely exposing the compound to the organophosphorous acid anhydrase (an enzyme produced via any process) does not necessarily effect any change to the organophosphorous compound. There is also no direct support for such discussion as found at pages 6-7 of the present brief on appeal in the present application written description. In fact page 8 states the inventors failed to achieve quantities of the enzyme needed. The discussion of the asserted (page 8-9) commercial scale is not demonstrated in the present application as filed nor contains any nexus to any part of the present written description. Here, in at page 9 of the brief, the summary asserts failure of others, however, such is not disclosed in the present specification. For the above reasons the summary as found in the brief is not concise nor correct since it diseases issues that are not supported by direct recitation in the present application.

Succinctly, the present invention defined by claim 53 which is a method for detoxifying an organophosphorous compound (generically recited and is any organophosphorous compound) by exposing the compound to the enzyme.

(6) Issues.

The appellant's listing (page 11 of the brief) of fifteen (15) items under "ISSUES" (appeal brief page 11) is noted, but is incorrect. There is but one issue - the appealed claims are not patentable. The claims fall together (see (5) Grouping of Claims below). The claims are not patentable because (A) the specification does not contain an adequate written description and enablement and (B) are rejected for that reason, (C) the claims are indefinite, and (D) the claims are obvious in view of the cited references, all of which lead to unpatentability.

(7) Grouping of Claims.

The statement at the bottom of page 11 of the present brief is noted as indicating that claims 53-64 are all properly of a single group. In view of that indication, and where appellant's brief contains no explicit statement that the claims stand or fall together, the claims, therefore, stand or fall together. See 37 CFR 1.192(c)(5).

(8) Claims Appealed.

The copy of the appealed claims contained in the appendix to the brief on appeal filed 16 August 1995 is incomplete because claim 64 is missing. The Supplemental Appeal Brief filed 24 November 1995 (for which appellant appears to have only filed one copy) contains a copy of claim 64 which was missing from the appendix of the appeal brief filed on 16 August 1995.

(9) Prior Art of Record.

The following is a listing of the prior art of record relied upon in the rejections of the claims under appeal.

BX Harper *et al.*, 1988, Appl. Environ. Microbiol. 54(10): 2586-2589.

Chaudhry *et al.*, 1988, Appl. Environ. Microbiol. 54(2): 288-293.

BY McDaniel *et al.* 1988, J. Bacteriol. 170(5): 2306-2311.

Mulbry *et al.* 1989, J. Bacteriol. 171(12): 6740-4746.

The Merck Index an Encyclopedia of Chemicals, Drugs, and Biologicals, 1983, (Windholz *et al.* eds.), Merck & Co., Inc., Rahway, NJ, page 1058.

Hawley's Condensed Chemical Dictionary, Twelfth Edition, (Lewis, ed.), Van Nostrand Reinhold Co., New York, page 857.

AT Wild *et al.* 1986, Proc. of the 1986 U.S. Army Chemical Research, Development and Engineering Center Scientific Conference on Chemical Defense Research, pages 629-634.

AZ McDaniel, C. S., 1985, "Plasmid-Mediated Degradation Of Organophosphate Pesticides", PhD Dissertation, pages iii to 164.

AW Munnecke, D. M. 1981, "The Use Of Microbial Enzymes For Pesticide Detoxification", in: Microbial Degradation Of Xenobiotics And Recalcitrant Compounds, Academic Press, Inc., London, Pages 251-269.

CD Munnecke, D. M., 1977, Appl. Environ. Microbiol. 33(3): 503-507.

Gottlieb, W., U.S. Patent 4,781,959 issued 1 November 1988, filed 1 August 1986.

Grot *et al.*, U. S. Patent 4,518,650 issued 21 May 1985, filed 7 August 1981.

The following reference are cited in regard to appellant's commentary in the summary of the invention and to define terms.

Stryer, L., 1975, in: Biochemistry, W. H. Freeman and Company, San Francisco, CA, page 197.

Webster's II New Riverside University Dictionary

Schulz *et al.* 1979, in: Principles of Protein Structure, Springer-Verlag, New York, pages 66-67

Watson *et al.* 1987, in: Molecular Biology Of The Gene, fourth edition, Benjamin/Cummings Publishing Company, Menol Park, CA, page 313 which factually states that all DNA is recombinant wherein the term does not therefore the term as used in the claims and argued in the brief, at for example in the paragraph bridging pages 36-37.

(10) New Prior Art.

No new prior art is applied in a rejection in this examiner's answer.

(11) Grounds of rejection.

The following ground(s) of rejection are applicable to the appealed claims.

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

The specification stands objected to under 35 U.S.C. 112, first paragraph, as failing to provide an adequate written description for practicing the claimed invention insofar as the present claims

indicate using a "recombinant" organophosphorous acid anhydrase. In the last full paragraph of page 39, appellant's brief asserts that there are four published versions of the *opd* gene of admittedly the same DNA where the present application disclosure is yet another sequence for the same DNA. The brief in this paragraph asserts that there is no as yet fully agreed upon sequence. Insofar as there is no admitted fully agreed upon sequence, it is not apparent that the sequence of the DNA from any reference not correct. Absent such factual side by side comparison, appellant has no basis to allege on the basis of the above admitted by appellant four published sequences for the identical DNA fragment especially where appellant wants to change the sequence of the DNA and amino acids for the organophosphorous anhydrase used in the presently claimed method to a sequence that contains unexplained and unaccounted for ambiguities. Such changes would be new matter and from the ambiguities present in sequence 6 of appellant's exhibit B, there is still not fully agreed upon sequence. Thus, the comments in appellant's brief do not even comply to the conditions for correction set forth in the *Ex parte Marshal* decision cited by appellant. It is pointed out that the specification recites using *P. diminuta* and a *Flavobacterium* sp (ATCC 27551) (see the cited references to Harper *et al.*, BX, and McDaniel *et al.*, BY) which set forth DNA sequences coding for *opd* where the organophosphorous acid anhydrase DNA set forth in Figure 1 of the specification are only partially identical. From the recited examples in the specification, it is not readily apparent that the species of bacteria are any different, nor that the plasmids used are any different, nor that the isolated DNA that was sequenced was any different, nor that the functionality encoded by the DNA is any different, and but yet the sequences recited in the Harper *et al.*, McDaniel *et al.*, Mulbry *et al.* (1) and Figure 1 of the specification set forth different DNA sequences coding for what is apparently the same enzyme. Note that page 21 of the specification recites using the plasmid pCMS1 (fig. 2 of Harper *et al.*) and sets forth the DNA sequence (fig. 1). This is apparently the same plasmid and DNA as in the specification (compare the paragraph bridging pages 23 and 24 of the specification and the McDaniel *et al.* reference, see RESULTS) which is expected to contain the identical DNA. The identical DNA should contain the same sequences not different sequences. Note also that fig. 4 of the McDaniel reference is identical to fig. 2 of the present application. Thus, there are apparently at least three different references all directed to the apparent identical genetic material where no one reference indicates a sequence identity for the apparently identical genetic material and therefore, a query is raised as to what genetic material is disclosed as coding for the organophosphorous acid anhydrase used in the

process of detoxification as each is the same but different and given that there are three disparate sequences from apparently the identical material (which disparity includes the DNA and amino acid sequence for the enzyme disclosed in the present application as files and as recited in the appealed claims), it is not clear that one of ordinary skill in the art using solely the disclosure in the application would have obtained the appropriate identical DNA encoding the organophosphorous acid anhydrase which is defined by the amino acid sequence of Figure 1. As appellant admits of not having the correct DNA and amino acid sequence at a time some several years after the instant application filing date.

Note in particular the indication in the response filed 28 October 1991 at page 15-16 indicating a 2% difference in sequence and the request to alter the sequence of Figure 1 (page 18). It is not clear what changes have been made in substitute Figure 1, as it is not apparently of record. It is noted that the above response cites *Ex parte Marsili et al.* among others (footnote, page 18-19 of the response), however, in *Marsili*, the specification was adequately enabling to support the change in formula of a chemical compound (note that neither a DNA polymer nor the amino acid sequence of a protein/enzyme is the same compound as an imidazole and the information content therein is radically different) whereas here, the process uses a recombinant enzyme defined by the DNA encoding the enzyme organophosphorous acid anhydrase where the specific amino acid sequence is a critical feature to the function of the enzyme. Here the specification and the response (filed 28 October 1991) alone do not show what changes applicant intends to make and whether or not those changes would have been adequately supported by the specification as originally filed, nor has any change been shown to have been an inherent characteristic of the enzyme used in the disclosed and presently claimed process. Thus, *Ex parte Marsili et al.* among others is not a definitive showing of the precedence of altering the DNA sequence of Figure 1 as originally filed in the instant application. Attention is directed to *Ex parte Fox*, 128 USPQ 157, (Pat Off Bd Appl, 1957) which indicated that where the name or formula (wherein the formula here is the seqence of bases in the DNA and the sequence of amino acids in the enzyme is a critical element) is a critical element in defining the instant invention alteration of that formula may constitute new matter when the original disclosure did not sufficiently identify the product or to permit positive identification so as to warrant the introduction of the newly discovered name (or formula). Here, the deletion, substitution resulting in a non-silent mutation, or insertion of a base results in a frame shift and there is no indication in the specification that the

corrections now furnished by applicant would have been deducible or anticipated from careful scrutiny of the specification. Note that in the modification of DNA, the resulting polypeptide product is not the same, especially in the instance of deletions, substitutions, and non-silent mutations.

The comments regarding page 15+ of the above 28 October 1991 response have previously been considered and are clearly indicative that the sequence as indicated in the application and those which have been published are disparate. Thus, the query of which sequence is correct still remains and given those disparities, it is apparent that the written description is flawed as the sequence comparison (Exhibit A) shows by indication of several hyphens, "-", defined as a "... base is missing ...", and, that the sequence as originally filed is incomplete as is evident from the comparative evidence of Exhibit A (see also the present marked copy of exhibit B attached as part of the examiner's answer). Note the numerous hyphens in the sequence indicated to be that which conforms to application figure 1. In the previous Office Action in the parent application, the specification was objected to because of the apparent disparity between the published sequences and the sequence set forth in the present application. In view of the disparities (Exhibit A filed with the response) and the request to correct the sequence shown in Figure 1, it is clearly apparent that the present application lacks an adequate written description for practicing the claimed invention with regard to the correct DNA sequence and the enzyme encoded by that DNA. Previously, Figure 1 of the present application was in one alternative the correct sequence, however, from Exhibit A (and exhibit B), it is clear that the sequence shown in Figure 1 in the present application is incorrect. Thus, the objection is not removed by the explanation and exhibit in appellant's response of 28 October 1991 and in view of the claims reciting a "recombinant organophosphorous acid anhydrase" used in the process where that organophosphorous acid anhydrase is defined by the sequence in Figure 1 and in view of the stated intention to correct Figure 1, the specification remains objected to.

The disparities (Exhibit A filed with the 28 October 1991 response) and the request to correct the sequence shown in Figure 1 clearly show the absence of an adequate written description for practicing the claimed invention with regard to the correct DNA sequence and amino acid sequence nor does the exhibit indicate whether or not such changes in the DNA affect the amino acid sequence in shown in Figure 1 of the present application which is in one alternative the correct sequence,

however, from Exhibit A, it is clear that the sequence shown in Figure 1 in the present application is considered incorrect by appellant. Thus, the objection to the specification is not removed by the explanation and exhibit in the response of 28 October 1991. In view of the present claims to a process using the recombinant enzyme and the intention to correct Figure 1, the objection is not seen as removable nor is it apparent as a minor correction in the absence of explanation of which amino acid sequence for organophosphorous acid anhydrase as coded for by the DNA sequence (the prior art or that of Figure 1 in the present application) is the correct sequence.

Insofar as the present specification discloses an enzymatic reaction resulting in degradation of the organophosphorous compounds by conversion into different product compounds, the present specification fails to disclose the process as occurring simply by exposing the enzyme to the compound. Note that simply "exposing" does not necessarily result in a detoxified compound as an organophosphorous compound which is not a substrate for the enzyme is not detoxified nor does "exposing" the compound to an inactive organophosphorous anhydrase enzyme result in a detoxified compound absent conditions effecting enzymatic conversion of substrate (the organophosphorous compound) into different product compounds or that the enzyme is effective to detoxify all organophosphorous compounds such as phosmet or phosphocreatine (see The Merck Index) both of which are organophosphorous compounds (i.e., organic compounds containing at least one phosphorous atom, see Hawley's Condensed Chemical Dictionary) as is DNA. Note that the present specification does not teach or disclose how to detoxify DNA or any other of a wide range of organophosphorous compounds.

Claims 53-64 stand rejected under 35 U.S.C. 112, first paragraph, for the reasons set forth in the objection to the specification.

Claims 53-64 stand rejected under 35 U.S.C. 112, first paragraph, as the disclosure is enabling only for claims limited to the specifically disclosed compounds such as parathion, paraoxon, and methyl parathion and the specifically disclosed enzyme as defined by the amino acid sequence shown in specification figure 1 (note the above objection to the specification) because the present specification discloses an enzymatic reaction resulting in degradation of the organophosphorous

compounds by conversion into different product compounds, the present specification fails to disclose the process as occurring simply by exposing the enzyme to the compound. Note that simply "exposing" does not necessarily result in a detoxified compound as an organophosphorous compound which is not a substrate for the enzyme is not detoxified nor does "exposing" the compound to inactive enzyme (as a result of proteolytic digestion or other adverse environmental physical, chemical, or biological parameter) result in a detoxified compound absent conditions effecting enzymatic conversion of substrate (the organophosphorous compound) into different compounds or that the enzyme is effective for detoxifying all organophosphorous compounds such as phosmet or phosphocreatine (see The Merck Index) both of which are organophosphorous compounds (i.e., organic compounds containing at least one phosphorous atom, see Hawley's Condensed Chemical Dictionary) as is DNA. Note that the present specification does not teach or disclose how to detoxify DNA (which is an organic compound that contains phosphorous) or any other of a wide range of organophosphorous compounds. See MPEP 706.03(n) and 706.03(z).

Claims 53-64 stand rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Claim 53 is incomplete as there is no stated result of the effect of exposing the compound with the organophosphorous acid anhydrase. Note that simply "exposing" does not necessarily result in a detoxified compound as an organophosphorous compound which is not a substrate for the enzyme is not detoxified nor does "exposing" the compound to inactive enzyme result in a detoxified compound. Insofar as the claims recite "organophosphorous compound" as noted in Hawley's Condensed Chemical Dictionary, the term refers to any compound containing carbon and phosphorous and is unclear as to whether or not the terminology is meant to be so inclusive as to include all organophosphorous compounds or whether it is meant to include only such compounds as parathion, paraoxon, and methyl parathion disclosed in the instant specification.

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section of the Examiner's Answer.

A person shall be entitled to a patent unless

(a) the invention was known or used by others in this country, or patented or described in a printed publication in this or a foreign country, before the invention thereof by the applicant for a patent; or,

(b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

The following is a quotation of 35 U.S.C. 103 which forms the basis for all obviousness rejections set forth in this Office Action:

> "A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.
>
> Subject matter developed by another person, which qualifies as prior art only under subsection (f) and (g) of section 102 of this title, shall not preclude patentability under this section where the subject matter and the claimed invention were, at the time the invention was made, owned by the same person or subject to an obligation of assignment to the same person.

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103, the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of potential 35 U.S.C. 102(f) or (g) prior art under 35 U.S.C. 103.

Claims 53, 54, 58, 59-63 stand rejected under 35 U.S.C. 102 (a) as anticipated by or, in the alternative, under 35 U.S.C. 103 as obvious over McDaniel *et al.* (BY) which discloses that organophosphorous acid anhydrase detoxifies organophosphorous compounds (see reference page 2306 and 2307) by conversion to products wherein the disclosed enzyme was obtained from a transformed microorganism. Here, the reference discloses cloning and expression of an *opd* gene encoding a phosphotriesterase using the same strains, vectors, restriction enzymes, and DNA fragment. Note the unexplained disparity of the sequences where given the fact that DNA is apparently the same DNA that was sequenced in the McDaniel *et al.* reference, the DNA is the same. In the alternative, given the starting materials and teachings in the McDaniel *et al.* reference, it would have been obvious that the ordinary skilled artisan would have obtained from using the disclosed probes,

DNA coding for the enzyme that was the same as that of the claims and used same in the disclosed process of degrading organophosphorous compounds.

Claims 53, 54, 58, 59-63 stand rejected under 35 U.S.C. 102 (a) as anticipated by or, in the alternative, under 35 U.S.C. 103 as obvious over Harper *et al.* (BX) which discloses (page 2586) the recombinant enzyme as degrading organophosphorous compounds wherein the enzyme was obtained from cloning and expression of an *opd* gene encoding a phosphotriesterase where the DNA sequence is the same for *P. diminuta* and a *Flavobacterium* sp (ATCC 27551). Note that the same strains, vectors, restriction enzymes, and DNA fragment are used in the present application and that there is an unexplained disparity of the sequences where given the fact that DNA is apparently the same DNA that was sequenced in the Harper *et al.* reference, the DNA is the same. Note the specification at page 11 lines 26-28 which states that "In another example the expression vector comprises a bacteriophage such as bacteriophage M13". In the alternative, given the starting materials and teachings in the Harper *et al.* reference, it would have been obvious that the ordinary skilled artisan would have, using the recited teachings, obtained the enzyme and used same in the process of degrading organophosphorous compounds.

Claims 53, 58, and 60 stand rejected under 35 U.S.C. 102 (b) as anticipated by Wild *et al.* (AT) who disclose exposing the organophosphorous acid anhydrase to organophosphorous compounds (see at least pages 629-630) as well as cloning and expression of DNA coding for organophosphate degrading enzymes from *P. diminuta* and a *Flavobacterium* which enzymes were used in the same disclosed process of degrading organophosphorous compounds.

Claims 61-63 stand rejected under 35 U.S.C. 102 (b) as anticipated by or, in the alternative, under 35 U.S.C. 103 as obvious over Wild *et al.* (AT) who disclose exposing the organophosphorous acid anhydrase to organophosphorous compounds (see at least pages 629-630) as well as cloning and expression of DNA coding for organophosphate degrading enzymes from *P. diminuta* and a *Flavobacterium*. In the alternative where the sequence is not disclosed, routine sequencing would have resulted in determination of the sequence of the cloned DNA which would have resulted in the amino acid sequence. Where the purified enzyme was sequenced, absent factual evidence to the

contrary, it would have been obvious that the enzyme disclosed in the reference has the same sequence as the enzyme in claims 61-63 and been used in the disclosed process of degrading organophosphorous compounds.

Claims 53, 54, and 60 stand rejected under 35 U.S.C. 102 (b) as anticipated by McDaniel (AZ) which discloses (see at least pages 45, 62, 101+) degradation (detoxification) of organophosphorous compounds using an enzyme obtained by cloning and expression of an *opd* gene encoding a phosphotriesterase using the same strains, vectors (such as pCMS1 which contains the DNA that is used in the presently claimed process and which upon sequencing has the DNA sequence of and encodes the enzyme with the amino acid sequence recited in the claims), restriction enzymes, and DNA fragment (see at least page iii, the tables, pages 46, 55-56, 69, figs. 17 and 19, 82, 89-91, and 116-120 and would have been the claimed process where the DNA encoded the enzyme used in the disclosed process of degrading organophosphorous compounds.

Claims 61-63 stand rejected under 35 U.S.C. 102 (b) as anticipated by or, in the alternative 35 U.S.C. 103 as obvious over McDaniel (AZ) who discloses (see at least pages 45, 62, 101+) degradation/detoxifying organophosphorous compounds using an enzyme obtained by cloning and expression of an *opd* gene encoding a phosphotriesterase using the same strains, vectors, restriction enzymes, and DNA fragment (see at least page iii, the tables, pages 46, 55-56, 69, figs. 17 and 19, 82, 89-91, and 116-120. It is pointed out that while the sequence is not disclosed, in the alternative and absent evidence to the contrary, routine sequencing would have resulted in determination of the sequence of the cloned DNA which would have led to the deduced amino acid sequence or where the purified enzyme was sequenced, absent factual evidence to the contrary, the enzyme disclosed in the reference has the same sequence as the enzyme in claims 61-63 and would have been the enzyme used in the disclosed process of degrading organophosphorous compounds.

Claims 53-54 and 59-64 stand rejected under 35 U.S.C. 103 as being unpatentable over Munnecke (AW) taken with Munnecke (CD), McDaniel *et al.* (BY) and Gottlieb (US '959).

Munnecke (AW) discloses processes using microbial enzymes in organophosphorous pesticide cleanup (see at least page 259) of containers, soil (page 260), and waste water (page 261) but where Munnecke (AW) do not set forth the organophosphorous compound in air, one of ordinary skill in the art would have from the citation of Munnecke (CD) by Munnecke (AW) have found it obvious to combine the disclosures of Munnecke (AW) with that of Munnecke (CD) which discloses detoxification of spray tank rinse water (page 507) wherein it would have been obvious to one of ordinary skill in the art to detoxify waste organophosphorous compounds in the aerosol spray (i.e. the compound is in the air). Moreover, where Munnecke (AW) disclose (page 258) that the enzyme was needed, it would have been obvious to one of ordinary skill in the art to combine the disclosures of Munnecke (AW) and Munnecke (CD) with that of McDaniel *et al.* (BY) which discloses organophosphorous acid anhydrase detoxification of organophosphorous compounds (see reference page 2306 and 2307) by conversion to products wherein the disclosed enzyme was obtained from a transformed microorganism. Here, where the reference discloses cloning and expression of an *opd* gene encoding a phosphotriesterase using the same strains, vectors, restriction enzymes, and DNA fragment. Given the starting materials and teachings in the McDaniel *et al.* reference, it would have been obvious that the ordinary skilled artisan would have obtained from using the disclosed probes DNA coding for the enzyme that was the same as that of the claims and which provides a source of the enzyme for use in the process disclosed by both Munnecke references and Gottlieb who discloses that such enzymes can be used to detoxify gaseous phase organophosphorous compounds (see at least col 3). Moreover, it would have been obvious by logical deduction from Gottlieb by one of ordinary skill in the art that organophosphorous degradation by the enzyme would have prevented contamination by pretreatment of an area with the enzyme which degrades the compound and that one known protective material is a gas mask. Thus, the claimed invention was within the ordinary skill in the art to make and use at the time it was made and was a whole, clearly *prima facie* obvious.

Claims 53-54 and 59-64 stand rejected under 35 U.S.C. 103 as being unpatentable over Munnecke (AW) taken with Munnecke (CD), and Wild *et al.* (AT) and Gottlieb (US '959).

Munnecke (AW) discloses processes using microbial enzymes in organophosphorous pesticide cleanup (see at least page 259) of containers, soil (page 260), and waste water (page 261)

but where Munnecke (AW) do not set forth the organophosphorous compound in air, one of ordinary skill in the art would have from the citation of Munnecke (CD) by Munnecke (AW) have found it obvious to combine the disclosures of Munnecke (AW) with that of Munnecke (CD) which discloses detoxification of spray tank rinse water (page 507) wherein it would have been obvious to one of ordinary skill in the art to detoxify waste organophosphorous compounds in the aerosol spray (i.e. the compound is in the air). Moreover, where Munnecke (AW) disclose (page 258) that the enzyme was needed, it would have been obvious to one of ordinary skill in the art to combine the disclosures of Munnecke (AW) and Munnecke (CD) with that of Wild *et al.* (AT) who disclose exposing the organophosphorous acid anhydrase to organophosphorous compounds (see at least pages 629-630) as well as cloning and expression of DNA coding for organophosphate degrading enzymes from *P. diminuta* and a *Flavobacterium* which enzymes would have been used in the disclosed process of degrading organophosphorous compounds set forth in both Munnecke references. Given the starting materials and teachings in the Wild *et al.* reference, it would have been obvious that the ordinary skilled artisan would have obtained enzyme that was the same as that of the claims and which provides a source of the enzyme for use in the process set forth by both Munnecke references and Gottlieb who discloses that such enzymes can be used to detoxify gaseous phase organophosphorous compounds (see at least col 3). Moreover, it would have been obvious by logical deduction from the Gottlieb patent by one of ordinary skill in the art that organophosphorous degradation by the enzyme would have prevented contamination by pretreatment of an area with the enzyme which degrades the compound and that one such piece of protective equipment was a gas mask. Thus, the claimed invention was within the ordinary skill in the art to make and use at the time it was made and was a whole, clearly *prima facie* obvious.

Claims 55-57 stand rejected under 35 U.S.C. 103 as being unpatentable over Munnecke (AW) taken with Munnecke (CD), McDaniel *et al.* (BY) and Gottlieb (US '959); or, under 35 U.S.C. 103 as being unpatentable over Munnecke (AW) taken with Munnecke (CD), and Wild *et al.* (AT) and Gottlieb (US '959) as applied to claims 53-54 and 59-64 above, and further in view of Grot *et al.* (US '650).

As both Munnecke references disclose detoxification of organophosphorous compounds using an enzyme and either of McDaniel *et al.* or Wild *et al.* disclose a process for obtaining that enzyme in

large quantities for the process disclosed in the both Munnecke references and in McDaniel *et al.* and Wild *et al.* which both disclose methods of and produced the enzyme and showed that the recombinant enzyme functioned to effect organophosphorous degradation, and where Gottlieb discloses using enzymes to detoxify gaseous phase organophosphorous compounds (see at least col 3); it would have been obvious by logical deduction from the Gottlieb patent by one of ordinary skill in the art that organophosphorous degradation by the enzyme would have prevented contamination by pretreatment of an area with the enzyme which degrades the compound and that one such piece of protective equipment was a gas mask wherein Grot *et al.* disclose masks that have been pretreated so as to provide protection from and detoxify at least in part organophosphorous compounds (see at least col 12 of Grot *et al.*). It would have been obvious to one of ordinary skill in the art to impregnate masks (disclosed in Grot *et al.*) for the purpose of detoxification of airborne organophosphorous compounds using the embedded enzymes disclosed in Gottlieb wherein said enzymes are those obtained by the process disclosed in either of McDaniel *et al.* or Wild *et al.* to effect a process such as disclosed in both of Munnecke references as modified by Grot *et al.* and Gottlieb *et al.* which disclose pretreating and embedding the materials to detoxify organophosphorous compounds wherein the method defined by the combined references would have been practices on a mask is a matrix which is a filtration device and is a gas mask. Thus, the claimed invention was within the ordinary skill in the art to make and use at the time it was made and was a whole, clearly *prima facie* obvious.

(12) New Ground(s) of rejection.

This examiner's answer does not contain any new ground of rejection.

(13) Response to Argument.

The commentary in the Appeal brief has been noted and considered but is not persuasive.

The objection to the specification under 35 U.S.C. 112, first paragraph, as failing to provide an adequate written description for practicing the claimed invention; and, the rejection of claims 53-64 under 35 U.S.C. 112, first paragraph, for the reasons set forth in the objection to the specification.

The comments in the response (pages 12-28, note that the appellant's discussion starts at page 12 and not at page 11 as indicated in the TABLE OF CONTENTS at page 1 of the brief) have been considered but are not persuasive. At page 12, the first item asserts that the specification presents the most accurate DNA sequence. This is not persuasive since appellant is seeking to change same. Thus, the comment as to accuracy in the application as originally filed is not persuasive and contradicted by appellant's present exhibit B in the brief.

The commentary spanning pages 12-13 indicated as the "A. The Examiner's Objection, the comments are noted but are not persuasive of appellant's position for the reasons indicated in the stated objection and rejection. Here, the comments by appellant are not persuasive because when the species of bacteria are the same, when the plasmid and the insert it contains are identical, the sequence of that polynucleotide should not differ. Appellant has provided no disclosure of what, how, or why, when and under what conditions as presently found in the application written description as originally filed that those differences are explained nor does appellant's desire to alter the sequence eliminate the issue since it presents yet another sequence in the exhibit B attached to the appellant's brief on appeal. It is appellant's application that presents the disparity. Appellant has not indicated any resequencing of deposited the material in question that has been obtained anew from a depository (appellant has not indicated that the new sequence in the exhibit B is from, for example, a resequencing of material from ATCC 27551), has not compared the material upon which the sequence is based (as disclosed in the present written description as originally filed) to any deposited material that is identical to that material upon which the present application written description is based, has not set forth any factual basis for errors in the present application written description of the sequence, has not set forth any explanation for how the error was discovered nor whether or not the such an error would or would not have been easily discovered by one skilled in the art.

In the last paragraph of page 12 and the first full paragraph of page 13, appellants continue their interpretation of the objection to the specification. Note that plasmid pCMS1 is pCMS1. When and where there are apparently three (3) different sequences for the identical DNA and the encoded amino acid sequence for the protein there is factual basis, when coupled with appellant's request to alter the sequence to yet another amino acid sequence different from any of the preceding amino acid

sequences (see appellant's exhibit B), to conclude that appellant's arguments are erroneous as the statement at page 12 of the brief that the DNA is the most accurate. If it was so accurate as filed, why do appellant's want to change same? The fact that appellant's want to change the sequence is directly contrary to appellant's statements of the most correct sequence having been presented. If the most accurate sequence has been presented why does appellant want to change same? This does not support appellant's position disclosure and inherency (see first 10 lines of page 12 of the present brief).

Appellant's remarks at page 13, second full paragraph have been considered but are not persuasive. The comments assert that one would have used ATCC 27551, however, page 7, lines 2-29 do not indicate any conditions of any deposit nor any indication of availability to the public for the practice of the invention. Thus, the disclosure of the sequence needs to be relied upon for the practice of the invention and the instant application written description in view of the objection does not provide an adequate written description since by appellant's admission of wanting to change the amino acid sequence, the DNA and the amino acid sequence present in the application as filed are incorrect. In this paragraph of the brief, appellant also acknowledges that others such as Serdar *et al.* suggest the change to the sequence of application figure 1. Thus, the comments in the second full paragraph of page 13 of appellant's brief are not persuasive.

In the last full paragraph of page 13 of appellant's brief, the comments are not persuasive as to the correctness of present application figure 1 - note the changes appellant wants to make to same. The further commentary as to changes being 2% at most is noted but is not persuasive as for example by simple arithmetic, there are some 48 changes (substitutions and missing bases) shown in 1356 bases (exhibit B) and this is more than 2% as argued in the brief as (48/1356)X100 = 3.5 and 3.5 is more than 2. Moreover, where these changes are indicated as suggested by others, this is not appellant's disclosure but of attributing these changes based upon the knowledge of others. Moreover, the changes shown in appellant's exhibit B in sequence 6 (the "corrected sequence") are not persuasive of correctness as it adds even more ambiguity since at about position 234, it is not clear whether or not the base is "G" or "C", at position 291, the "?" is representative of the fact that appellant does not even know what or whether or not there should even be a base present. See also positions 1164, 1165, 1169, 1194, 1195, 1201, 1202, 1249-1251, 1266, and 1267 as to the fact that appellant

does not even know what or whether or not there should even be a base present. Moreover, note the exhibit B hyphens "-" which are indicated as missing bases in some sequences but present in others. See for example positions 7-9, 353-356, 704, 707, and 1147 where these bases would be absent from the corrected sequence. Thus, the comments in this paragraph of the brief are not persuasive.

The commentary (brief page 14, first full paragraph) refers to references by McDaniel *et al.*, Harper *et al.*, Mulbry *et al.*, and Serdar *et al.* as to whether or not they are prior art. The comments are noted but are not persuasive under 35 U.S.C. 112 first paragraph since the criterion is not the references and whether or not they are or are not prior art. The criterion is appellant's written description in the application. Thus, the comments are not persuasive nor germane to the criteria of 35 U.S.C. 112 which indicates in part that it is the specification shall contain a written description of the invention and from the foregoing and the stated objection to the specification, it is apparent that the present application written description does not contain an adequate written description since the DNA encoding the amino acid sequence (exhibit B, sequence 6) which appellant wants to use in place of the present application figure 1 (exhibit B, sequence 1) sequence is not and was not in the possession of appellant at the time of the effective filing date of the present application.

In the second full paragraph of page 14, the brief admits of the sequences as being different, however, as to the McDaniel *et al.* (BY) reference the plasmid pCMS1 is the plasmid containing the relevant DNA, and in the Harper *et al.* reference, the relevant polynucleotide is from the same pCMS1 plasmid, and in the Mulbry *et al.* (J. Bacteriol., 1989) reference, it is the identical bacterium - ATCC 27551 from which the DNA was obtained. Thus, it is not apparent that the identical DNA should have different sequences. In this paragraph, appellant also asserts that it is a gross misstatement to characterize the sequences as only partly identical. This is noted but is not persuasive in view comparison in appellant's exhibit B. Rather it is appellant who has created the differences to which the present application seeks to alter the originally filed sequence but which changes have no basis or support in the written description of the application as originally filed. Insofar as this paragraph directs ones attention to exhibit B, such consideration of the exhibit does not support any of appellant's assertions. Here, the issue is not whether or not the sequences are or are not overwhelmingly identical (exhibit B clearly shows they are not, and what is and was indicated at one point in the

prosecution of the present application is that the written description is flawed since the same DNA should have the same sequence, not a different sequence - on that basis the written description is flawed), it is whether or not the present application written description supports the changes appellant wants to make. Based upon the written description in the present application as filed, the comments in appellant's prior responses and the present brief do not support appellant's changes and therefore, the comments in the second paragraph of the brief are not persuasive.

In the paragraph bridging pages 14-15, it is noted that the paragraph asserts there are 1430 bases however, the instant exhibit B only indicates 1356 bases and it is not clear where or what bases are or are not the (1430-1356=74) 74 bases that are referred to but not shown in exhibit B. In fact, it is apparent from this paragraph of the brief that appellant is still confused as to the correct (i.e., the most accurate sequence), thus, it is not apparent that even in sequence 6 of appellant's brief that appellant has or has not the correct sequence. The discussion of the changes at 9 different positions (McDaniel *et al.*) and the subsequent inventors is stated to correlate to a percentage difference of "less than 1.0% (0.63%), however, it is apparent that when some (48/1356)X100 = 3.5) 48 bases are changed, the difference is 3.5%. Appellant first argues 2% (brief page 13 at the bottom) then argues less than one percent (paragraph bridging pages 14-15) but clearly neglects to explain that 48 bases are changed or substituted and even at (48/1430)X100=3.35%) 1430 bases, the percentage change is 3.35% which is clearly more than the less than one percent argued. Thus, the comments are not persuasive and are not supported by the present application written description as originally filed.

The comments in the first full paragraph of page 15 discusses the 35 U.S.C. 112 requirement for disclosure of the best mode. Insofar as appellant asserts that the inventors were making every attempt to meet that criterion, the comments are noted but are not commensurate to the above indicated objection to the specification which is not predicated upon the absence of a best mode. The objection is based as indicated above upon inadequate written description. Thus, the comments as to best mode are not persuasive to remove the objection and rejection as the objection and rejection are not predicated upon absence of best mode. This paragraph also asserts the present figure 1 as an improvement of the published sequences, however, there is no disclosure that the sequence is an improvement. Moreover, when appellant's changes in sequence 6 of exhibit B are considered, it is

apparent that introduction of more ambiguity is not an improvement. Here the numbers presented in this paragraph of the brief indicate now that there are 17 out of 1174 bases or 12 out of 1248 bases however, as previously argued in the brief there are 1430 bases. What is the correct number? The arguments in this paragraph of the brief do not clarify any issue, rather is presents more confusion based upon appellant's arguments because it makes more unclear as to what changes are correct and more unclear as to how many bases and amino acids are really intended for the claims. In this paragraph the present brief cites *In re Bosy* (footnote at page 15) in connection with the Harper *et al.* reference as to best mode but is not persuasive since the objection is not one of absence of best mode but of absence of adequate written description to which appellant's reliance upon *In re Bosy* is misapplied since the decision does not appear to address nor is the issue of optimum mode an issue in the stated objection and rejection. Thus, commentary in this regard using the Mulbry *et al.*, Harper *et al.*, and Serdar *et al.* references is not persuasive of appellant's position for the above indicated reasons since the objection and rejection are not predicated upon absence of a best mode but on the absence of an adequate written description.

In the paragraph bridging pages 15-16, it is noted that appellant asserts that the sequence is difficult to accurately obtain since the G+C content is high and refers to the Serdar *et al.* reference. The comments are noted, however, difficult does not mean not possible and even where difficult, the art is replete with examples of genes and amino acids that have been sequenced. Note that here, the claimed method uses a protein - i.e., it has an amino acid sequence and an amino acid sequence is not a DNA sequence. Thus, the commentary regarding the G+C content is not persuasive as in this instance the G+C content refers to the DNA. The DNA is not the protein/enzyme and the G+C base content is not an amino acid or a sequence of amino acids. It is also noted that this paragraph also cites the Sedar *et al.* reference as to the "G" and "C" ratio, however, the Serdar *et al.* reference is not *per se* the instant written description and therefore, is not persuasive. Thus, the comments in the paragraph bridging pages 15-16 are not persuasive.

In the first paragraph of page 16, refers to changes of "G" and "C" in the sequences, however, none of these demonstrate that the sequence 6 shown in the present exhibit B to which applicant wants to change the present application figure 1 sequence to was in the possession of appellant at the

time of filing of the present application nor does the present application disclose nor suggest that any of the "G" and "C" bases should be changed. Thus, the specification does not support any changes that appellant seeks to make and the comments in the first full paragraph of page 16 are not persuasive.

It is noted that the second paragraph of page 16 cites *Ex parte Marsili* which is not persuasive since the present application facts and fact pattern differ from *Ex parte Marsili*. It is also noted that appellant discusses the decision in *Ex parte Maizel*.

It is noted that the paragraph bridging pages 16-17 to the paragraph bridging pages 17-18 discusses and presents appellant's interpretation of the *Ex parte Marsili* decision. However, the present facts differ in at least three respects because the present invention is (1) not a claim to a compound - it is to a method; (2) the changes that appellant wants to make are not minor modifications as is clear from the above paragraphs; (3) the compound discussed in the *Ex parte Marsili* is not a protein. It is also noted that the brief discusses the *In re Nathan* decision which cites the *Petisi et al. v. Rennhard et al.* decision. Here appellant asserts erroneous statement, but where appellant has presented a declaration filed concomitant with the application that indicates that appellant has read and understood the contents and claims of the application it is not apparent that appellant can argue an erroneous statement. However, it is apparent from appellant's written description as filed does not support the changes appellant now wants to make. The changes to the nucleotide base and amino acid sequence are not corrections to the formula nor has appellant demonstrated that the changes are inherent. Thus, the discussion of *Ex parte Marsili* in the brief does not support appellant's position.

It is noted that the first full paragraph of page 18 to the end of the paragraph ending at the top of page 24 discusses and presents appellant's interpretation of the *Ex parte Maizel* decision in regard to the new matter, the objection to the specification, and the rejection of the claims. In the paragraph bridging pages 21-22, it is noted that appellant comments that appellant was unable to accurately sequence the DNA. This is exactly the case here as is plainly evident from appellant's discussion in the present brief. Moreover, here, it is readily apparent that appellant was also unable to accurately sequence the protein - else why does appellant want to change the sequence. Furthermore, it is also readily apparent that appellant is still unable to accurately sequence the protein because the changes

that appellant wants to make (attention is directed to sequence 6 of exhibit B) clearly demonstrates that appellant does not know what amino acids are or are not present insofar as the DNA sequence is incompletely/inaccurately sequenced so that the amino acid sequence is not accurately known. See for example bases where there are "-" and "?". Thus, the situation is exactly similar to *Ex parte Maizel*.

It is also noted that appellant presents commentary regarding the DNA encoding the enzyme, however, here, the claim is a process of detoxifying organophosphorous compounds using an enzyme used in the process of detoxifying organophosphorous compounds that is recited in product by process format. The enzyme is a protein. It is not the DNA. The process by which the enzyme is obtained does not alter the process of detoxifying organophosphorous compounds. In view of appellant's commentary and the changes appellant wants to make to the DNA it is not apparent that the appellant had the correct amino acid sequence. It is not apparent that appellant now has the correct sequence nor can the sequence disclosed in the application as filed and in sequence 6 of exhibit B be considered correct in view of appellant's assertions in the present brief on appeal as sequence 6 of exhibit B contains changes that are not disclosed in the application as originally filed nor are the changes appellant wants to make inherent in the application disclosure as originally filed. In fact there are positions in the sequence for which appellant now places question marks, i.e., positions for which the base is now unidentified. Thus the sequence as disclosed and the sequence to which appellant wants to change the sequence to are not accurate and any changes as set forth sequence 6 in appellant's exhibit B is at least as inaccurate as the original sequence of figure 1 of the patent application.

Appellant provides further four comments (top half of page 24) regarding the present application in comparison to *Ex parte Marsili*. As to the first comment that the appellant discovered minor errors, this not readily apparent from the present prosecution history - rather it is apparent that such "minor errors" but which are not minor errors were pointed out in the Office Action mailed 24 May 1991 (paper number 4). Thus, it is evident that appellant did not find the error nor is it apparent from the discussion at page 24 of the appellant's brief where in the specification as originally filed these errors are referred to. As to the second comment that that analytical data/literature support the requested changes, the comments are not persuasive for the reasons indicated above. Moreover, it is not the references that must support the changes, it is appellant's written description in the present

application as originally filed which application does not demonstrate the presence of the DNA and the amino acid for sequence 6 of exhibit B in the application as originally filed nor where nor how sequence 6 of exhibit B is obtained solely from the present application as originally filed. Thus, the second point at page 24 (top half of the page) is not persuasive. As to the third comment of that there are sufficient characteristics to distinguish the compound, the comment is not persuasive as the proposed changes (exhibit B, sequence 6) contain more ambiguity than the originally filed DNA and amino acid sequences shown in application figure 1. Moreover, appellant's admit the originally filed sequences contain errors but do not disclose how they arose, the conditions that effected their discovery (as it is apparent from paper 4 that appellant did not appear to have found such errors). Appellant has not apparently gone back and resequenced any deposited material nor compared same to the originally filed sequences. There is no comparison of the protocols used in the original as opposed to that for the changes (sequence 6 of exhibit B) that appellant wants to make. Thus, the comment 3 at page 24 (top half of the page) is not persuasive. As to point 4, appellant argues that there is no threat of adding new matter. This is noted because the changes would constitute new matter which is not entered. Thus, the comments comparing the present application to that of the *Ex parte Marsili* decision are not persuasive since the facts differ.

In the bottom half of page 24 to the top one-third of page 25 appellant presents eight comments that attempt but do not distinguish present application from that of the *Ex parte Maizel* decision. As to the first comment that the changes are minor, the comment is not persuasive since

as filed (a)	ATG met 1(1)	CAA gln	ACG thr	AGA arg	AGG arg	GTT val	GTG val	CTC leu	AAG lys	TCT ser
exhib. B (b)	ATG met 1(1)	CAA gln	ACG thr	AGA arg	AGG arg	GTT val	GTG val	CTC leu	AAG lys	TCT ser
as filed (a)	GCG ala 31(11)	GCC ala	GCA ala	GGA GLY	ACT THR	CTG LEU	CTC LEU	GGC GLY	... ...	
exhib. B (b)	GCG ala	GCC ala	GCC *ala	GCA ALA	GGA GLY	ACT THR	CTG LEU	CTC LEU	... ...	

31(11)
The 1(1) and 31(11) are the base and amino acid numbers respectively

Starting at amino acid number 14, the sequence diverges and contains added amino acids to the entire sequence. It is exacerbated as one continued through the modified sequence. Thus, the comment in the response that the changes are not major are not persuasive as it cannot even be determined from the present application whether or not the modifications that appellant makes in sequence 6 of exhibit B include or exclude the active site residues (the modification of which would clearly be expected to have a major impact upon enzymatic function and activity.

It is also noted that appellant asserts no protein sequence is claimed. This is not persuasive since to practice the claimed method, one needs the enzyme. The enzyme which is defined by its amino acid sequence since the sequence defines the primary, secondary and tertiary structure of a protein (see Schulz *et al.* at for example page 67 which is cited as common knowledge in the art). Claim 61 among other claims recites a sequence for the protein and therefore since the claimed process relies upon the enzymatic function of the protein, it is evident that a protein and its correct sequence are necessary. Thus, contrary to appellant's comments the claims do contain in the claims a sequence of amino acids for a protein which is the enzyme. This makes the appellant's first comment in the bottom half of page 24 regarding the *Ex parte Maizel* decision incorrect and erroneous.

The second and third comments in the bottom half of page 24 assert that there is ample description for the invention to distinguish same in the claims. The comments are noted but are not persuasive because the modifications that appellant wants to make are not supported by the present application. The sequence in the present application as originally filed as effectively admitted in the present brief and prior responses is incorrect and results in conclusion of an inadequate written description. It is not apparent that an admittedly wrong sequence (figure 1 as filed) and another sequence (sequence 6, exhibit B) which presents additional ambiguities and undisclosed in the present application as filed changes is any more correct than the originally filed incorrect amino acid and DNA sequence. As to the third point that the DNA is described, so noted, however, it is admitted in appellant's brief as incorrectly set forth as originally filed. Moreover, as pointed out above, where appellant's brief refers to cells deposited with the ATCC, the material was not obtained anew from the

depository nor resequenced nor any conditions set forth for the comparison of the newly sequenced material as compared to that used in producing the original sequence. Insofar as appellant alleges a unique sequence, where the sequence admittedly contains errors as filed and contains undefined bases was well as additional bases that include entire codons the shift the reading frame. Thus, it is apparent that where appellant argues uniqueness, it is uniquely incorrect and inaccurate as filed. Thus, appellant's comment as to the second and third points at the bottom half of page 24 in the present brief are not persuasive of distinctness from the *Ex parte Maizel* decision.

In point 4 (bottom of page 24) appellant's brief asserts purification and to the extent possible sequenced portions of the protein and argues that the plasmid produces a complete native protein. The comment is noted but not persuasive in view of the present claims which do not indicate the enzyme is purified. The comment is also not persuasive since as pointed out above, the sequence is incorrect. Thus, the comments as to the item 4 are not persuasive.

It is noted that the item 5 at the top of brief page 25 asserts that there is only one molecule having organophosphorous acid anhydrase activity. So noted. This also makes points 1-4 (bottom of brief page 24) inaccurate and unpersuasive since where there is purportedly only one sequence, it is not apparent from appellant's exhibit B that they even have the correct sequence since sequence 6 contains additional ambiguities.

In item 7 (note that there is no item 6 as the brief contains no item 6 in this regard) at brief page 25, appellant comments that the claims describe the DNA. This is not persuasive. The claims are to a process of detoxifying any organophosphorous compound. The process uses an enzyme. An enzyme is a protein. A protein is not a DNA regardless of whether a DNA is used to make the protein, the process uses a protein to effect "exposing". Furthermore, appealed claims 61-63 contain recitation of the amino acid sequence that is admittedly incorrect to encode the enzyme which is the compound used in the process that effects the detoxification. The DNA does not *per se* effect any detoxification. Thus, appellant's comments are not persuasive.

Item 8 (brief page 25) asserts that the invention claimed uses a specific composition and is not a generic invention directed to DNA. This is true. The claimed process uses the enzyme. The enzyme is a protein. The enzyme is not a DNA. Thus point 8 does is not persuasive.

In each of points 1-5, 7, and 8 (there is no point 6 in appellant's discussion spanning pages 24-25) appellant argues that the facts differ from *Ex parte Maizel* decision. For the reasons indicated above, appellant's comments are not persuasive because (1) appellant seeks to make changes to the sequence that are not trivial, not disclosed in the application as originally filed, and are not supported by the original disclosure; (2) the changes contain substitutions and insertions where the insertions and substitutions result in a modified protein to which all of item (1) in this paragraph are applicable; (3) there is no comparison of the conditions for the originally filed sequence and that which is presently proffered by appellant (sequence 6, exhibit B) where in the present application it is not readily apparent that the functional and enzymatic activity of the protein is not altered - note that the present application does not even indicate which amino acid residues are or are not involved in catalysis, substrate binding, or that which affects protein folding, the pH profile, the temperature profile, etc. and thus, it is not apparent that the changes appellant wants to make can be said with any degree of certainty to be minor; (4) there is no analytical data for any resequencing nor any analytical data supporting the changes appellant wants to make that are described in the application as originally filed; (5) the description of the DNA and therefore of necessity, the protein (since the protein is encoded by the DNA) is admittedly incorrect where the issue as broached is that in the original disclosure, there is no support for the changes appellant wants to make but where the application figure 1 DNA and amino acid sequence are incorrect and like the *Ex parte Maizel* decision, appellant admits the sequences differ but also argue that there is but one sequence and it is apparent that appellant was not in the possession of sequence 6 exhibit B when the parent of the present application was filed. Thus, appellant's comments regarding the present application and the *Ex parte Maizel* decision are not persuasive of a difference.

The comments at page 25 under "The Claimed Invention" are noted but are not persuasive because where the claims are to methods, they use the enzyme regardless of how it is made. The sequence of the DNA and the amino acids recited in claim 61 that is referred to in this section of the

brief, requires the enzyme to have the specific sequence, not the putative sequence that is alleged. Insofar as the amino acid sequence is what defines the enzyme, the sequence is admittedly incorrect for the reasons indicated above and because appellant is seeking to change same (if it is not wrong, why is appellant changing same?) As to claim 53, the enzyme recited therein is a protein and it is defined only by function. It is not defined by the DNA nor is a DNA inherent to a protein as proteins exist in the absence of a DNA. For example, the enzyme can be obtained from the organism that naturally produces it or it can be made via traditional chemistry which does not rely upon a DNA. Thus, the appellant's discussion of the claimed invention at page 25 does not support any of appellant's position.

In the paragraph "The Description of the DNA" bridging pages 25-26, the brief asserts the methods do not require the amino acid sequence. The comments are not persuasive because a protein is what has the enzymatic function and activity, the DNA does not. Insofar as appellant makes the allegation that no specific amino acid sequence is required, it is erroneous because one cannot have a protein with no amino acid sequence and is unsupported by any factual scientific evidence that one can use a DNA to make a protein that has no amino acid sequence. Moreover, the sequence of DNA as originally filed is incorrect as admitted by appellant in the brief and the correction contains changes that are not supported by the present application and its parent application as originally filed. Thus, the present facts are not like the *Ex parte Marsili* decision but are like the *Ex parte Maizel* decision for the above indicated reasons.

The two full paragraphs of page 26 allege the errors in the DNA are sequence are minor and refer to exhibit B. All of the foregoing paragraphs and the discussion of appellant's exhibit B have been considered and for the reasons indicated above, the comments regarding minor changes are not persuasive. In fact it is apparent in the first full paragraph of page 26 that appellant is still not even sure of the accuracy of the amino acid sequence of the exhibit B (see sequence 6).

In the last full paragraph appellant requests the best way to correct the sequence. The following and other criteria have been used in evaluation of appellant's comments and materials presented for modification of the sequence as originally filed. First appellant needs to have a correct

sequence with no blanks and no question marks. Secondly, appellant needs to compare the originally filed DNA and in this case also the amino acid sequence of the protein against that DNA that has been obtained anew from the depository and to show a chain of custody so that there is a certainty that it is the same DNA and the same protein that is described in the application that was obtained from the depository. Thirdly, appellant needs to indicate that the deposited material and the DNA and amino acid sequences obtained therefrom are accurate (as appellant's previous attempts have been admitted as incorrect) since the present written description is admitted by appellant as inaccurate. Fourth, appellant needs to demonstrate that there is no difference in function by comparing all of the chemical, physical, biological, and enzymatic functions of that which is disclosed as originally filed as compared to that which is purported to be the same. Fifth, appellant needs to demonstrate where in the present application as originally filed (i.e., in the parent application) the changes are supported by the application written description especially since the present application at pages 7-8 assert the DNA is invariant (specification pages 21-22, 24-25 and example VIII do not even suggest any differences). The same DNA must encode the same protein. Therefore, it is apparent that appellant here, like the appellant in the *Ex parte Maizel* decision, does not have the DNA nor the protein nor the correct sequence of both the DNA and the protein that is the enzyme which is used in the process. Thus, the comments in the two full paragraphs of page 26 are not persuasive.

In the first two full paragraphs of brief page 27, appellant comments that the DNA is precisely 1.3 kb, however, the comments are not persuasive in view of appellants comments in the brief as for example, brief page 15 asserts there are 1248 bases and 1248 bases is not 1.3 kb (1,300 bases). It is also asserted at page 15 of the brief that the DNA is 1430 bases and 1430 bases is 130 bases more than appellant's assertion of 1300 bases. In fact it is apparent that from appellant's exhibit B that appellant considers 1356 bases as well. Thus, it is apparent that appellant does not consider 1300 bases as the exact size of the DNA. In fact appellant's second paragraph of page 27 argues that the 1.3 kb is not exact. Thus, the comments are not persuasive.

Appellant third full paragraph of page 27 argues that there is but one enzyme. So noted. Insofar as there is only one sequence as asserted by appellant, the appellant's exhibit B does not demonstrate that appellant has the correct amino acid.

In the fourth full paragraph of page 17 to the end of the first full paragraph of page 28, it is noted that the brief asserts that there is a foolproof way to obtain the DNA and its exact sequence. This is noted but is not persuasive. Where appellant has followed the teachings in the present application and indicates that it is foolproof and provides the exact sequence, then there is no need to correct the sequence yet appellant here seeks to change the sequence. Therefore appellant's methods are not foolproof as is plainly evident from appellant's own comments.

> The rejection of claims 53-64 under 35 U.S.C. 112, first paragraph, as the disclosure is enabling only for claims limited to the specifically disclosed compounds such as parathion, paraoxon, and methyl parathion and the specifically disclosed enzyme as defined by the amino acid sequence shown in specification figure 1 (note the above objection to the specification).

The comments in the response (pages 28-29) have been considered but are not persuasive. The comments in the paragraph bridging pages 28-29 refer to page 32, 33, and 31, lines 16+, 20+, and 20+ respectively in the present application as to the types of organophosphorous compounds, however, the present claims are not defined solely by these compounds since the enzyme is not effective to detoxify all organophosphorous compounds. See phosmet and/or phosphocreatine (see The Merck Index) both of which are organophosphorous compounds (i.e., organic compounds containing at least one phosphorous atom, see Hawley's Condensed Chemical Dictionary). DNA also contains numerous phosphate groups and is an organophosphorous compound as defined by Hawley's Condensed Chemical Dictionary (see page 857) which indicates that "organophosphorous compound" is "Any organic compound containing phosphorous as a constituent" and includes nucleic acids. Thus, appellant's claim terminology is not limited to only the compounds argued in the appeal brief.

It is also noted that the paragraph bridging pages 28-29 and the first full paragraph of page 29 assert commercialization of the enzyme, however, commercialization is not a criterion of adequate written description that enables. The present application does not contain any definition of the term organophosphorous which definition is therefore that found in the Hawley's Condensed Chemical Dictionary. Insofar as the specification is asserted in appellant's brief as not addressing "each and

every" (which is redundant) substrate, the instant application does not indicate nor disclose nor define the above terminology used in the claim. The assertion of "key substrates" is noted but neither the claims nor the specification define "key substrates" *per se*. Thus, the comments are not persuasive. Attention is also directed to appellant's later comments in the brief admitting of no commercial market.

In the first full paragraph of page 29, it is noted that the brief asserts that appellant knows of no way to list "each and every" (redundant as each is every and every means all which is each) substrate, however, the instant claims (53-64) do not even indicate the organophosphorous compound is a pesticide. As pointed out above, the present specification does not teach or disclose how to detoxify DNA (which is an organic compound that contains phosphorous) or any other of a wide range of organophosphorous compounds. Thus, the comments in the response are not persuasive.

The rejection of claims 53-64 under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

The comments in the response (pages 29-30) have been considered but are not persuasive. In the last full paragraph of page 29, the brief asserts that appellant's have used "exposing" in place of "contacting", "interacting", "reacting", etc., however, "exposing" appears to have been defined as "to deprive of shelter or protection" or "to subject or allow to be subjected to an action or an influence" or "to make visible" or "to make known" or "to reveal the guilt or wrongdoing of". None of these refer to "contacting" (a union or junction of surfaces), "interacting" (to act on each other), "reacting" (i.e., to act in response to or under the influence of a stimulus or prompting). None of "exposing" suggests the words "contacting", "interacting", "reacting" as argued by appellant. Attention is directed to the definitions provided in Webster's II New Riverside University Dictionary. The further discussion of poorer choice of words such as "mixing", "dissolving", and "solvating" are noted, however, basic enzymology terminology such as "... catalyzes the reaction ..." is known. Thus, it is readily apparent that the claims are not defined by "exposing".

In the paragraph bridging pages 29-30, the brief refers to the term "organophosphorous" as objected to and provides exhibits F-K in support thereof. The comments are not, however, persuasive

because the claims recite "organophosphorous compound" which as set forth in Hawley's Condensed Chemical Dictionary, refers to any compound containing carbon and phosphorous and is unclear as to whether or not the terminology is meant to be so inclusive as to include all organophosphorous compounds or whether it is meant to include only such compounds as parathion, paraoxon, and methyl parathion disclosed in the instant specification. The reliance upon exhibits F-K from the Environmental Protection Agency do not define the terminology of organophosphorous compound nor are the exhibits F-K part of the present application as originally filed. Moreover, the fact sheets do not contain *per se* any definition of the terminology. Thus, reference and reliance upon the exhibits F-K is not persuasive nor is the assertion of substrate for the enzyme (page 30) persuasive since the present claims do not indicate *per se* that the "organophosphorous compound" is a substrate for the enzyme. Thus, the comments in the brief are not persuasive.

The rejection of claims 53, 54, 58, 59-63 under 35 U.S.C. 102 (a) as anticipated by or, in the alternative, under 35 U.S.C. 103 as obvious over McDaniel *et al.* (BY)

The comments at page 30 under "A. The Examiner's Rejection" are noted as appellant's summary of the stated rejection. It is not persuasive of appellant's position. In part "B. Appellants' Remarks", (paragraph spanning pages 30-31) comment upon *Ex parte Hirschler*, *In re Katz*, and *In re Kusko* in regard to disclaiming affidavits but misapplies each.

Insofar as appellant's brief indicates that discussion of obviousness under 35 U.S.C. 103 is treated separately. That discussion is apparently found at page 36 of the present brief under what appellant refers to as "ISSUE V." The same comment is presented, i.e., that the reference is not prior art. This argument is not persuasive for the reasons indicated in the immediately preceding paragraphs because and until applicant filed the appropriate disclaiming affidavits, the reference is by another to the present inventive entity and is published prior to the present application effective filing date and for the reasons indicated in the stated ground of rejection, the reference if not anticipatory, makes obvious the present claims on appeal.

In the first full paragraph of page 31 (and including the paragraph bridging pages 31-32 and the footnote of pages 31), the remarks refer to the "Declaration of Invention" filed in conjunction with the present application as a "sworn satisfactory explanation" of the inventorship and argues that Raushel was not an author and that Harper was a technician and Miller was a student. Suffice to say, the instant declaration that is referred to by appellant McDaniel is not a disclaiming affidavit under any of *Ex parte Hirschler*, *In re Katz*, and *In re Kusko* nor does that declaration state anything with regard to Harper or Miller nor does it antedate any reference. The present inventive entity is McDaniel, Raushel, and Wild whereas the reference is by McDaniel, Harper, and Wild. When the names of McDaniel and Wild are removed from the reference, the name of Harper remains and the name of Raushel is missing from the reference. The reference is therefore by another and was published in 1988 which is prior to the present application effective filing date of 27 April 1989. Thus, the reference qualifies as prior art under 35 U.S.C. 102 (a) which indicates the invention was known or used by others (the authors of the reference are not identical to the present inventive entity) in this country, or patented or described in a printed publication (the reference is a printed publication that is published in a major peer reviewed journal that is readily available and indexed so as to expedite any search for the reference) in this or a foreign country (the reference was published in the United States), before the invention thereof (the reference was published prior to the effective filing date) by the applicant for a patent by another. Thus, appellant misapplies the decisions. Appellant has provided no disclaiming affidavit that conforms to the facts of *Ex parte Hirschler* or *In re Katz*. Insofar as appellant has in the parent application indicated willingness to file such a declaration, appellant has not done so. Therefore, the comments in appellant's brief are not persuasive. Thus, the reference qualifies as prior art under 35 U.S.C. 102 (a) which indicates the invention was known or used by others (the authors of the reference are not identical to the present inventive entity) in this country, or patented or described in a printed publication (the reference is a printed publication that is published in a major peer reviewed journal that is readily available and indexed so as to expedite any search for the reference) in this or a foreign country (the reference was published in the United States), before the invention thereof (the reference was published prior to the effective filing date) by the applicant for a patent by another.

The rejection of claims 53, 54, 58, 59-63 under 35 U.S.C. 102 (a) as anticipated by or, in the alternative, under 35 U.S.C. 103 as obvious over Harper *et al.* (BX)

The comments at page 32 under "A. The Examiner's Rejection" are noted as appellant's summary of the stated rejection. It is not persuasive of appellant's position.

In part "B. Appellants' Remarks", (paragraph spanning pages 32-33) assert that the Harper *et al.* reference was published in October of 1988 and that the present application was filed in April of 1989. The comment is noted and is demonstrative that the reference was published prior to the present application. In the paragraph bridging pages 32-33, appellant comments again upon the explanation of authorship and inventorship which is not persuasive. The present inventive entity is McDaniel, Raushel, and Wild whereas the reference is by Harper, McDaniel, Miller, and Wild. When the names of the presently named inventors is removed from the list of authors of the reference, the names of Harper and Miller remain and the name of Raushel is missing from the reference. Thus, this reference is also legally by another to the present inventive entity.

Insofar as appellant's brief (page 32) indicates that discussion of obviousness under 35 U.S.C. 103 is treated separately. That discussion is apparently found at page 36 of the present brief under what appellant refers to as "ISSUE VII." The same comment is presented, i.e., that the reference is not prior art. This argument is not persuasive for the reasons indicated in the immediately preceding paragraphs because and until applicant filed the appropriate disclaiming affidavits, the reference is by another to the present inventive entity and is published prior to the present application effective filing date and for the reasons indicated in the stated ground of rejection, the reference if not anticipatory, makes obvious the present claims on appeal.

The rejection of claims 53, 58, and 60 under 35 U.S.C. 102 (b) as anticipated by Wild *et al.* (AT)

The comments at page 33 under "A. The Examiner's Rejection" are noted as appellant's summary of the stated rejection. In particular, appellant comments upon routine sequencing is needed

to obtain the sequence. So noted. However, it is also pointed out that since the presently rejected claims do not recite a specific sequence, any sequence of DNA that encodes the enzyme is all that is needed and any protein of any amino acid sequence that has the function is all that is needed wherein the reference certainly discloses that there has been obtained the DNA and the protein that perform the function. It is not persuasive of appellant's position.

In part "B. Appellants' Remarks", (paragraph spanning pages 33-34 to the end of the first full paragraph of page 34) assert that the Wild *et al.* reference does not teach the DNA sequence of the gene nor anticipate any difficulty the present inventors encountered. The comments are not persuasive. The discussion of the open reading frame and no disclosed DNA sequence is noted but not persuasive because the rejected claims 53, 58, and 60 do not require nor recite any specific DNA sequence and therefore any DNA that encoded the protein is effective and sufficient to produce the protein which has no amino acid sequence recited as used in the claimed process. The discussion of 250 bases and DNA from the 5' flanking DNA is also not persuasive for the above indicated reasons and is not commensurate in scope to the presently rejected claims. Moreover, where appellant alleges that removal of the 250 bp fragment "eliminates" any OPA activity, this is not supported by appellant's present specification which in the paragraph bridging pages 24-25 that it is "without complete loss of activity", i.e., there is biological activity. This statement in appellant's application is the antithesis of appellant's statement in the brief of "eliminates" any OPA activity. The argument (page 34) of teaching removal of a region of the *opd* gene and reference to figure 2 are not persuasive because the presently rejected claims 53, 58, and 60 do not require nor recite the removal of DNA. The claims are directed to a method of using an enzyme. The enzyme is a protein and is composed of amino acid residues. It is not a DNA. Thus, appellant's comments are not persuasive nor is the Wild *et al.* reference confusing nor does it so state. Thus, the comments in appellant's brief are not persuasive.

Insofar as appellant's brief (page 33) indicates that discussion of obviousness under 35 U.S.C. 103 is treated separately, claims 53, 58, and 60 in this rejection are rejected for anticipation. Thus, the comment regarding separate discussion for obviousness is not persuasive nor is it apparently discussed in the present brief. Thus, the comment is not persuasive.

The rejection of claims 61-63 under 35 U.S.C. 102 (b) as anticipated by or, in the alternative, under 35 U.S.C. 103 as obvious over Wild *et al.* (AT)

Appellant's brief contains no apparent discussion of this rejection under 35 U.S.C. 102 (b). It is therefore, a defective brief on appeal (see 37 C.F.R. 1.192 (c) (8) (iii)), however, it is also taken as agreement that the claims are anticipated because there is no discussion traversing the above indicated rejection for anticipation.

As to the comments at page 36 regarding the obviousness rejection under 35 U.S.C. 103, the discussion asserts the Wild *et al.* reference teaches away from the claimed invention but does not indicate how or what is relied upon for support of the assertion made. As pointed out above in the ground of rejection, the Wild *et al.* reference discloses exposing the organophosphorous acid anhydrase to organophosphorous compounds (see at least pages 629-630) as well as cloning and expression of DNA coding for organophosphate degrading enzymes from *P. diminuta* and a *Flavobacterium*. In the alternative where the sequence is not disclosed, routine sequencing would have resulted in determination of the sequence of the cloned DNA which would have resulted in the amino acid sequence. Where the purified enzyme was sequenced, absent factual evidence to the contrary, it would have been obvious that the enzyme disclosed in the reference has the same sequence as the enzyme in claims 61-63 and been used in the disclosed process of degrading organophosphorous compounds. Thus, appellant's comments at page 36 are not persuasive.

The rejection of claims 53, 54, and 60 under 35 U.S.C. 102 (b) as anticipated by McDaniel (AZ)

The comments at page 34 under "A. The Examiner's Rejection" are noted as appellant's summary of the stated rejection and is not persuasive of appellant's position.

In part "B. Appellants' Remarks", (first two full paragraphs of page 35) assert that the reference is appellant's PhD dissertation and is argued as preliminary. The reference does however, disclose the claimed process as set forth in the above indicated ground of rejection. Appellant argues that the DNA sequence for the *opd* gene is not disclosed. This is not persuasive as the plasmid pCMS1

contains same and the sequence is inherent to the gene, however, the rejected claims do not require nor recite any specific DNA sequence. Insofar as appellant professes difficulties in sequencing the DNA and/or the enzyme, the comments are not persuasive because present specification pages 22, 24, and 25 recite utilization of known and routine procedures for sequencing (i.e., done for example according to the manufacturers instructions is not demonstrative of any difficulty) nor does the instant application indicate difficulties in sequencing. Thus, it is appellant's own application written description that does not support appellant's assertions in the present brief. In the second full paragraph of page 35, appellant's comment that the reference does not teach how to make the invention is clearly erroneous because it discloses how to conduct the process - i.e., treating organophosphorous compounds with an OPD enzyme where the enzyme is encoded by DNA in pCMS1 (which is the identical DNA in the present application written description that contains the DNA) and the reference teaches that the organophosphorous compounds are degraded (i.e., detoxified). Thus, appellant's comments are in error and are not persuasive.

Insofar as appellant's brief (page 34 last full paragraph) indicates that discussion of obviousness under 35 U.S.C. 103 is treated separately, claims 53, 54, and 60 in this rejection are rejected for anticipation. Thus, the comment regarding separate discussion for obviousness is not persuasive nor is it apparently discussed in the present brief. Thus, the comment is not persuasive. Insofar as appellant professes difficulties in sequencing, the comments are not persuasive because present specification pages 22, 24, and 25 recite utilization of known and routine procedures for sequencing (i.e., done for example according to the manufacturers instructions is not demonstrative of any difficulty) nor does the instant application indicate difficulties in sequencing. Thus, it is appellant's own application written description that does not support appellant's assertions in the present brief.

The rejection of claims 61-63 under 35 U.S.C. 102 (b) as anticipated by or, in the alternative 35 U.S.C. 103 as obvious over McDaniel (AZ)

The comments (last full paragraph of page 35 to the end of the paragraph bridging pages 35-36) in the brief have been considered but are not persuasive. Appellant argues that the DNA sequence of the opd gene is not disclosed and argues that sequence information is/was not available.

The comments are not persuasive as for example the present brief (page 34) admits that sequencing was done. As pointed out above, where appellant professes difficulties in sequencing, the comments are not persuasive because present specification pages 22, 24, and 25 recite utilization of known and routine procedures for sequencing (i.e., done for example according to the manufacturers instructions is not demonstrative of any difficulty) nor does the instant application indicate difficulties in sequencing. Thus, it is appellant's own application written description that does not support appellant's assertions in the present brief. Appellant argues that the reference does not anticipate, however, appellant has presented no factual scientific evidence demonstrating any difference of the DNA encoding the enzyme and the enzyme produced therefrom as used in the process wherein organophosphorous compounds are detoxified as put forth in the reference as compared to the DNA in pCMS1 that encodes the enzyme. Therefore, the comments in the brief are not persuasive.

At page 36, the discussion in the brief in what appellant indicates as "ISSUE XII.", the comment that McDaniel teaches away in important regards is noted but is not persuasive for the reasons indicated in the stated ground of rejection and for the above indicated reasons.

The rejection of claims 53-54 and 59-64 under 35 U.S.C. 103 as being unpatentable over Munnecke (AW) taken with Munnecke (CD), McDaniel *et al.* (BY) and Gottlieb (US '959)

The comments at the bottom of brief page 36 and the paragraph bridging pages 36-37 have been considered but are not persuasive. The comments refer to Munnecke I and II, but does not make it clear which is reference I and which is reference II. In that regard, the Munnecke references will be referred to as Munnecke (AW) taken with Munnecke (CD). Contrary to appellant's assertions, the Munnecke AW and CD references do much more than just allude to treating pesticides, it disclosed processes using microbial enzymes in organophosphorous pesticide cleanup (see at least page 259) of containers, soil (page 260), and waste water (page 261) and states that the enzyme as is here recited in the appealed claims is used in processes for detoxification of organophosphorous compounds.

As to the Munnecke (CD) reference it appears that appellant would argue that it only indicates a crude enzyme. This is noted but is not persuasive. No appealed claim even calls for using a purified enzyme and appellant's allegations would appear to read limitations into the claims that are not present in the appealed claims. Here, the bacterial culture produces at least an enzyme that effects the detoxification reaction and is produced from and encoded by DNA within the bacterial cells wherein all DNA is recombinant. Thus, appellant's assertions are not persuasive.

As to the comment regarding the McDaniel *et al.* reference, appellant makes the allegation that the reference is not prior art but is not a persuasive argument because the reference is legally by another to the present inventive entity, was published prior to the present application effective filing date. Moreover, as indicated above, where appellant has commented that the "Declaration of Invention" filed in conjunction with the present application is a "sworn satisfactory explanation" of the inventorship. The reference is cited qualifying art under 35 U.S.C. 102 (a) where the criteria are (1) by another (there is a difference between the named inventors and the named authors which is a different entity from McDaniel, Raushel, and Wild. Suffice to say, there is no declaration that is referred to by appellant McDaniel that meets the criteria set forth in *Ex parte Hirschler*, *In re Katz*, and *In re Kusko* since there is no disclaiming affidavit from any person who is not a named inventor and there is no disclaiming affidavit from any person who is a named inventor who states in the appropriate declaration format set forth in the *In re Katz* decision, and, the citation of *In re Kusko* is noted but is misapplied by appellant since the reference is applied as art under 35 U.S.C. 102 (a) and 35 U.S.C. 102 (a) is not 35 U.S.C. 102 (f). There is no declaration stating anything with regard to Harper or Miller nor does it antedate any reference. The present inventive entity is McDaniel, Raushel, and Wild whereas the reference is by McDaniel, Harper, and Wild. When the names of McDaniel and Wild are removed from the reference, the name of Harper remains and the name of Raushel is missing from the reference. The reference is therefore by another and was published in 1988 which is prior to the present application effective filing date of 27 April 1989. Thus, the reference qualifies as prior art under 35 U.S.C. 102 (a) which indicates the invention was known or used by others (the authors of the reference are not identical to the present inventive entity) in this country, or patented or described in a printed publication (the reference is a printed publication that is published in a major peer reviewed journal that is readily available and indexed so as to expedite any search for the reference) in this or a

foreign country (the reference was published in the United States), before the invention thereof (the reference was published prior to the effective filing date) by the applicant for a patent by another. Insofar as appellant has in the parent application indicated willingness to file such a declaration, appellant has not done so. Therefore, the comments in appellant's brief are not persuasive. Thus, the reference qualifies as prior art under 35 U.S.C. 102 (a) which indicates the invention was known or used by others (the authors of the reference are not identical to the present inventive entity) in this country, or patented or described in a printed publication (the reference is a printed publication that is published in a major peer reviewed journal that is readily available and indexed so as to expedite any search for the reference) in this or a foreign country (the reference was published in the United States), before the invention thereof (the reference was published prior to the effective filing date) by the applicant for a patent by another.

In the paragraph bridging pages 36-37, appellant alleges that the Munnecke I (presumed to be Munnecke (AW)) does not refer to a recombinant enzyme. This comment is noted but is not persuasive as all DNA is recombinant and therefore, the proteins encoded thereby are also recombinant. Thus, assertions regarding recombinant or not recombinant are not persuasive. As discussed above and in the stated ground of rejection, the comments such as general reference as to what appears to be the Munnecke (AW) reference is not persuasive. As to the Munnecke II (presumably the Munnecke (CD) reference, attention is directed to the stated ground of rejection and the above comments in the preceding paragraph which indicate that appellant's comments are not persuasive because as pointed out, no claim even requires a purified enzyme. The claims do not even contain the word. This paragraph then asserts that Gottlieb merely alludes to immobilized enzyme, however, the appellant's comment is not persuasive because it discloses that such enzymes can be used to detoxify gaseous phase organophosphorous compounds (see at least col 3) where, in view of the combined references, it would have been obvious by logical deduction from the Gottlieb and the remaining cited references to one of ordinary skill in the art that organophosphorous degradation by the enzyme would have prevented contamination by pretreatment of an area with the enzyme which degrades the compound and that one known protective material is a gas mask. Thus appellant's comments are not persuasive. The iteration of the comment by appellant that the McDaniel *et al.*

reference as not being prior art is noted but for all of the foregoing reasons that comments by appellant is not persuasive because it is wrong.

The rejection of claims 53-54 and 59-64 under 35 U.S.C. 103 as being unpatentable over Munnecke (AW) taken with Munnecke (CD), and Wild *et al.* (AT) and Gottlieb (US '959).

The comments in what appellant calls "ISSUE XIV" are noted but are not persuasive for the reasons indicated above with regard to Munnecke (AW) taken with Munnecke (CD), and Wild *et al.* (AT) and Gottlieb (US '959). It is noted that appellant asserts that Wild *et al.* teaches away from the invention for the above indicated reasons. Appellant's "above indicated reasons" were not and remain unpersuasive as set forth in the preceding paragraphs of this examiner's answer. It is also pointed out that the presently rejected claims (in part) do not recite a specific sequence, any sequence of DNA that encodes the enzyme is all that is needed and any protein of any amino acid sequence that has the function is all that is needed wherein the reference certainly discloses that there has been obtained the DNA and the protein that perform the function. It is not persuasive of appellant's position. As to appellant's prior comments regarding the obviousness rejection under 35 U.S.C. 103, the discussion asserts the Wild *et al.* reference teaches away from the claimed invention but does not indicate how or what is relied upon for support of the assertion made. As pointed out above in the ground of rejection, the Wild *et al.* reference discloses exposing the organophosphorous acid anhydrase to organophosphorous compounds (see at least pages 629-630) as well as cloning and expression of DNA coding for organophosphate degrading enzymes from *P. diminuta* and a *Flavobacterium*. In the alternative where the sequence is not disclosed, routine sequencing would have resulted in determination of the sequence of the cloned DNA which would have resulted in the amino acid sequence. Where the purified enzyme was sequenced, absent factual evidence to the contrary, it would have been obvious that the enzyme disclosed in the reference has the same sequence as the enzyme in claims 61-63 and been used in the disclosed process of degrading organophosphorous compounds. Thus, appellant's comments at page 37 are not persuasive.

The rejection of claims 55-57 under 35 U.S.C. 103 as being unpatentable over Munnecke (AW) taken with Munnecke (CD), McDaniel *et al.* (BY) and Gottlieb (US '959); or, under 35 U.S.C. 103 as being unpatentable over Munnecke (AW) taken with Munnecke (CD), and Wild *et al.* (AT) and Gottlieb (US '959) as applied to claims 53-54 and 59-64 above, and further in view of Grot *et al.* (US '650).

It is noted that appellant presents that same arguments regarding the Munnecke (AW) and (CD) references, the Wild *et al.* reference, and the McDaniel *et al.* references. Appellant's comments are not persuasive for the reasons indicated above. The reasons are applied here as indicated above as to each of the Munnecke, Wild *et al.*, and McDaniel *et al.* references. It is noted that the appellant asserts that the references alone or combined do not teach or suggest the claimed invention but presents no reasoning in support thereof and is there fore not persuasive.

In the paragraph bridging pages 37-38, it is noted that appellant's brief discusses the Grot *et al.* reference by asserting that there is no teaching of using an organophosphorous degrading enzyme. The comments are not persuasive as all DNA is recombinant and therefore any protein produced therefrom is also recombinant. Moreover, from the combined references, one of ordinary skill in the art that organophosphorous degradation by the enzyme would have prevented contamination by pretreatment of an area with the enzyme which degrades the compound and that one such piece of protective equipment was a gas mask wherein Grot *et al.* disclose masks that have been pretreated so as to provide protection from and detoxify at least in part organophosphorous compounds (see at least col 12 of Grot *et al.*). It would have been obvious to one of ordinary skill in the art to impregnate masks (disclosed in Grot *et al.*) for the purpose of detoxification of airborne organophosphorous compounds using the embedded enzymes disclosed in Gottlieb wherein said enzymes are those obtained by the process disclosed in either of McDaniel *et al.* or Wild *et al.* to effect a process such as disclosed in both of Munnecke references as modified by Grot *et al.* and Gottlieb *et al.* which disclose pretreating and embedding the materials to detoxify organophosphorous compounds wherein the method defined by the combined references would have been practices on a mask is a matrix which is a filtration device and is a gas mask. For these reasons, appellant's comments are not persuasive.

At page 38 of the appeal brief, appellant presents more commentary that is not addressed to any one specific rejection under 35 U.S.C. 103 and cites the *Graham v. John Deere* decision as to the criteria set forth for determining obviousness.

As to the scope and content of the prior art, appellant's brief again discusses the McDaniel *et al.* reference (paragraph bridging pages 38-39) by assertion of appellant's prior comments as sufficient to overcome the rejection. The commentary is noted but as pointed out above in the discussion of the claims 53, 54, 58, 59-63 under 35 U.S.C. 102 (a) as anticipated by or, in the alternative, under 35 U.S.C. 103 as obvious over McDaniel *et al.* (BY), appellant's comments are and remain unpersuasive for the above indicated reasons as applied here.

In the first full paragraph of page 39, appellant's brief again discusses the Harper *et al.* reference as not prior art and refers to appellant's reasons regarding the McDaniel *et al.* reference. The comments are not persuasive for the reasons indicated above in the discussion of the claims 53, 54, 58, 59-63 under 35 U.S.C. 102 (a) as anticipated by or, in the alternative, under 35 U.S.C. 103 as obvious over McDaniel *et al.* (BY), appellant's comments are and remain unpersuasive for the above indicated reasons as applied here. Moreover, appellant's comments are also not persuasive for the reasons set forth in the ground of rejection of claims 53, 54, 58, 59-63 under 35 U.S.C. 102 (a) as anticipated by or, in the alternative, under 35 U.S.C. 103 as obvious over Harper *et al.* (BX) and as explained in this section in response to appellant's comments which are applied here as indicated above.

Appellant's discussion of the Wild *et al.* reference (item c, spans the bottom half of page 39 to the end of the first full paragraph of page 43) has been considered but is not persuasive.

The assertion that the reference does not teach the sequence of the *opd* gene is not persuasive since claims 53-60 do not require nor recite any specific sequence of a DNA used to encode the enzyme nor any specific amino acid sequence for the enzyme used in the process and where a sequence is recited, appellant has not demonstrated by factual scientific side by side comparison that the DNA that is disclosed in the Wild *et al.* reference does not contain the identical as

now claimed or identical as appellant would now like to claim as sequence 6 of exhibit B. Here, Wild *et al.* have and did disclose the DNA that encoded the enzyme and used same to effect detoxification of organophosphorous compounds. Whether or not the DNA and the enzyme are sequenced, the sequence provides "information" and does not *per se* alter the composition of the DNA nor of the enzyme used in the process disclosed in the Wild *et al.* reference which process is that of the present claims.

As to the assertion of not anticipating the difficulty the present inventors had in obtaining the sequence and initiation codon for expression, the comments are not persuasive because as discussed above, difficult is not the same as unsuccessful. Moreover, the cited reference is a demonstration of successful examples of cloning the gene which is the antithesis of appellant's assertion. Moreover, no claim *per se* recites any manipulation of the gene *per se* that is not obvious to do since the Wild *et al.* reference manipulates same as disclosed in the reference. As to the allegation of critical expression, the comment is noted but is not persuasive since there is no indicated criticality of expression recited in the claims nor is it apparent from appellant's written description as filed. Moreover, it is readily apparent that the DNA disclosed in the Wild *et al.* reference contained an initiation codon since Wild *et al.* obtained expression of a protein that effected detoxification of organophosphorous compounds where it is known that for expression to occur in a bacterial system, that the DNA needs to have an ATG or the alternative start codon. Thus, appellant's comments are not persuasive as it would have been obvious to anyone of ordinary skill in the art that there was an intiation codon present.

As to the allegation that Wild *et al.* does not teach a means for overcoming any difficulties encountered by the inventors, the comments are not persuasive because the present application does not contain any indication of any difficulties encountered nor even suggest that there are any. In fact, appellant's written description appears to indicate that only routine procedures have been used as the present specification pages 22, 24, and 25 recite utilization of known and routine procedures for sequencing (i.e., done for example according to the manufacturers instructions is not demonstrative of any difficulty) nor does the instant application indicate difficulties in sequencing. Thus, it is appellant's own application written description that does not support appellant's assertions in the present brief.

Regarding the allegation of teaching away from the present invention, the comments are not persuasive. The comment does not indicate how or what is relied upon for support of the assertion made. As pointed out above in the ground of rejection, the Wild *et al.* reference discloses exposing the organophosphorous acid anhydrase to organophosphorous compounds (see at least pages 629-630) as well as cloning and expression of DNA coding for organophosphate degrading enzymes from *P. diminuta* and a *Flavobacterium*. In the alternative where the sequence is not disclosed, routine sequencing would have resulted in determination of the sequence of the cloned DNA which would have resulted in the amino acid sequence.

Appellant also alleges that there is nothing to suggest the combination, however, the comment is not persuasive because where Munnecke (AW) disclose (page 258) that the enzyme was needed, it would have been obvious to one of ordinary skill in the art to combine the disclosures of Munnecke (AW) and Munnecke (CD) with that of Wild *et al.* (AT) who disclose exposing the organophosphorous acid anhydrase to organophosphorous compounds (see at least pages 629-630) as well as cloning and expression of DNA coding for organophosphate degrading enzymes from *P. diminuta* and a *Flavobacterium* which enzymes would have been used in the disclosed process of degrading organophosphorous compounds set forth in both Munnecke references. Given the starting materials and teachings in the Wild *et al.* reference, it would have been obvious that the ordinary skilled artisan would have obtained the enzyme that was the same as that of the claims and which provides a source of the enzyme for use in the process set forth by both Munnecke references and Gottlieb who discloses that such enzymes can be used to detoxify gaseous phase organophosphorous compounds (see at least col 3). Moreover, it would have been obvious by logical deduction from the Gottlieb patent by one of ordinary skill in the art that organophosphorous degradation by the enzyme would have prevented contamination by pretreatment of an area with the enzyme which degrades the compound and that one such piece of protective equipment was a gas mask. Thus, one of ordinary skill in the art would clearly have recognized and used the combined references as all are directed to the same field of art, to the same invention, to the same subject matter, and to the solution of the same problem.

For the above reasons, the items (1) through (4) in the item (c) of appellant's brief at page 39 are not persuasive.

In the last full paragraph of page 39, appellant's brief asserts that there are four published versions of the *opd* gene of admittedly the same DNA (note the reference disclosure of 1300 bases which is the same number of bases that appellant asserts encode the enzyme) where the present application disclosure is yet another sequence for the same DNA. The brief in this paragraph asserts that there is no as yet fully agreed upon sequence. Insofar as there is no admitted fully agreed upon sequence, it is not apparent for the above indicated reasons that the sequence of the DNA from the Wild *et al.* reference is incorrect. Absent such factual side by side comparison, appellant has no scientific comparison of the DNA disclosed in the Wild et al. reference to allege the DNA is not the same/identical DNA. Thus, appellant's comments in the last full paragraph of page 39 are not persuasive.

In the first full paragraph of page 40, appellant's brief comments that there was difficulty in obtaining the translational open reading frame for the *opd* gene. The comment is not persuasive since Wild *et al.* demonstrated obtaining the enzyme that effected detoxification of organophosphorous compounds that was produced from transcription and translation of a DNA in a transformed microorganism. This also demonstrates that contrary to the alleged "handful" of *Pseudomonas* genes, that this particular gene was and had been obtained wherein the DNA sequence of the disclosed DNA in the reference does not alter *per se* the DNA polynucleotide disclosed in the reference (the sequence (i.e., the information contained in the DNA) is an inherent part of the DNA. Moreover, the Wild *et al.* reference indicates (page 632) that "the same procedure has been utilized to enhance the expression of the initially cloned Flavobacterium gene (10)". Thus, the comments in this paragraph of appellant's brief is not persuasive.

The commentary in the second full paragraph of page 40 in appellant's brief is noted as to the admitted known ATG and GTG start codons and is persuasive that the disclosed DNA in the combined cited references which would have had the appropriate ATG start codon. As to the allegation of totally different codons for start codons, the suppositions in the brief are unfounded in any scientific or legal facts of record that is supported by the present application as originally filed (unexpected properties and other secondary considerations of success as argued by appellant in the present brief are not

supported by the instant application written description - see *In re Davies and Hopkins*, 177 USPQ 381 (Fed. Cir. 1973), which indicates that while unexpected properties can be used to show unobviousness, those unexpected properties must be found in the application as filed and appellant's inability to correctly sequence the DNA and the protein is not an unexpected property of the DNA nor of the protein, rather it may be an unexpected property of appellant and appellant is not the claimed invention). Thus, the comments in appellant's brief are not persuasive.

In the paragraph bridging pages 40-41, it is noted that appellant's brief asserts difficulties and failed to sequence the enzyme. Note that sequencing the enzyme to determine its amino acid sequence (the representation of the information content of the enzyme using symbolic notation) does not *per se* alter the enzyme (a composition of matter). It is also noted that the brief refers to specification page 9 as indicative of problems encountered in obtaining amounts of material of sequencing. The comment is noted but not persuasive because page 9 refers not to amounts of material but to "activity". Activity is not an amount of enzyme. Moreover, as to the Wild *et al.* reference, it is indicated therein that the enzyme is secreted into the culture medium (see page 629 and 632-633 of the Wild *et al.* reference which uses an inducible system for expression) and therefore, assertions regarding effective amounts of membrane associated enzyme as a necessity are not persuasive because the reference indicates that the membrane form of t he enzyme has been kept frozen for months without appreciable loss of activity - i.e., one has a large quantity of the enzyme since it can be stored for months and multiple batches of the enzyme can be combined. It is also noted that appellant's brief refers to page 23, lines 10-34 and page 25, lines 8-13 as support for using fusion proteins, however, in view of the foregoing, and the present application written description of using only routine recognized protocols for sequencing, it is not apparent that using fusion protein is a necessity.

In the first full paragraph of page 41, the brief asserts the Wild *et al.* reference is a preliminary report that does not teach the open reading frame. The comments are noted but are not persuasive since the reference discloses the process of detoxifying organophosphorous compounds using a recombinant enzyme wherein the process which produced the enzyme used a DNA that contained an open reading frame else the reference would have successfully obtained the enzyme product used in the process disclosed in the reference. The discussion of removing 250 bases and using PstI sites is

also noted, however, the Wild *et al.* reference indicates that this is not the sole construct for expression of the DNA encoding the enzyme encoded by the 1300 bases. Thus, the comments in the brief are not persuasive.

At page 42, it is noted that appellant's brief cites (and discusses) the *Amgen Inc. v. Chugai Pharmaceutical Co. Ltd.* decision and in the paragraph bridging pages 42-43 asserts that the amino acid sequence for the organophosphorous anhydrase was not known. The comments are noted but are not persuasive because for certain of the claims, no particular amino acid sequence for the enzyme is recited in the claims and where a DNA/amino acid sequence is recited, appellant has not demonstrated nor compared the DNA encoding the enzyme and the enzyme disclosed in the Wild *et al.* reference to that of the present application claims on appeal which appeal exhibit B is demonstrative of errors in appellant's sequence (and is admitted to by appellant in prior responses and in the present brief). Thus, it is readily apparent that appellant does not and did not have a complete mental picture of the DNA nor of the amino acid sequence for the enzyme nor knew whether or not the DNA and the amino acid sequences were correct. Thus, the present application does not demonstrate that appellant had the complete mental picture of the invention since even now appellant's exhibit B does not contain a DNA or an amino acid sequence for the enzyme that contains no ambiguities. Where appellant admits of not having the complete or correct sequence, it is apparent that there is no complete mental picture.

In the first full paragraph of page 43, it is noted that appellant concludes that on the basis of additional ambiguities (exhibit B) that the Wild *et al.* reference should be removed. The comments are not persuasive for the reasons indicated in the stated ground of rejection and for the reasons in the preceding paragraphs of the examiner's answer.

It is also noted that there is no item d at page 43 of appellant's brief. It skips directly to item e and discusses the McDaniel (AZ) reference. Appellant argues the reference is preliminary, however, there is no indication in the reference of appellant's alleged "preliminary" nature. Appellant's arguments that no sequence is present in view of difficulties is noted but is not persuasive since this reference contains the identical DNA from pCMS1 which contains the DNA that encodes the enzyme.

Appellant has not demonstrated that the DNA disclosed in the reference is not the same in sequence and does not have the same sequence of amino acids in the enzyme. Appellant argues various unnamed and unspecified difficulties, however, appellant's own application written description indicates that only routine amino acid sequencing protocols were needed to obtain the sequence. Thus, comments regarding the McDaniel (AZ) reference are not persuasive as it is readily apparent that even the present exhibit B contains an inaccurate DNA and therefore an inaccurate amino acid sequence. Thus, the comments in the brief are not persuasive.

In the first full paragraph of page 44 it is noted that the response asserts a serious error in the sequencing and refers to table 9 on page 100, and discusses the start site. The discussion of what the skilled technician would not have used is noted but is not persuasive since pCMS1 can be used and the reference does in fact demonstrate that the enzyme was obtained via expression of the DNA present in pCMS1 or various other constructs made therefrom. Moreover, the reference discloses the DNA the effects the enzymatic function of organophosphorous detoxification and regardless of whether or not the DNA was sequenced (sequencing only adds information about the DNA it does not change the sequence of bases of the DNA that is being sequenced) one has the DNA of the claims where appellant's present written description admits of only using routine known protocols for effecting the sequencing. Thus, the comments are not persuasive.

In the paragraph bridging pages 44-45, appellant refers to page 98, lines 21-24 and page 101, lines 1-2. Appellant argues that sequencing would require S1 nuclease mapping and or purification. These comments are noted, however, in order to use the DNA one does not necessarily need the sequence as the McDaniel (AZ) reference among other cited references clearly shows that others had also obtained expression of the DNA (without knowledge of the sequence *per se*) that resulted in the product enzyme. In any event, the issue is not the sequence of the DNA for the enzyme as what is claimed is a process of detoxifying organophosphorous compounds using an organophosphorous anhydrase which the reference clearly discloses. Here, appellant has not demonstrated that the DNA encoding the enzyme and the amino acid sequence of the enzyme are different from that set forth in the claims wherein appellant's present exhibit B is a clear demonstration that appellant still cannot

define the correct sequence since sequence 6 of exhibit B contains ambiguities. Thus, the comments in the paragraph bridging pages 44-45 are not persuasive.

The first full paragraph of page 45 is noted as directing attention to the absence of a molecular weight for the enzyme disclosed in the reference. The comments are noted but are not persuasive and not commensurate in scope to the claims. No claim requires not recites *per se* any molecular weight. Moreover, molecular weight is relative and is dependent upon the conditions used when estimated by chromatographic methods. Appellant's calculations in this paragraph are noted and correlate to the molecular weight of what would have been expected from a 1300 base pair fragment of which appellant admits that there was a band of about 65 kD. Insofar as appellant asserts that there are other bands, the comments are noted but not persuasive the molecular weight was not known as appellant has demonstrated that such is easily calculated, however, one would not have indicated that the 10 kD molecular weight was appropriate from appellant's calculations, rather one would have logically used the 65 kD value since it is mathematically correct. Thus, the comments in the brief are not persuasive

The paragraph bridging pages 45-46 in the brief asserts that it does not teach one how to make the invention. The comments are not persuasive because the reference discloses constructing plasmids used in transformed bacteria (i.e., recombinant DNA) which is used to produce the enzyme (i.e., a recombinant enzyme) and used the enzyme in a process that effected the detoxification of organophosphorous compounds - see the stated ground of rejection. Thus, appellant's comments as to the McDaniel (AZ) reference as not anticipatory is not persuasive nor does the reference teach away from the claims as for example the rejected claim 53 is fully met by the reference.

In the first full paragraph of page 46, appellant's brief asserts that both Munnecke references, the Gottlieb and Grot *et al.* references do not discuss methods of using recombinant enzymes (note that all DNA is recombinant and therefore all proteins produced from such DNA is also a "recombinant" enzyme. Here where appellant states a referral to their previous arguments, it is pointed out that appellant's comments as discussed above are not persuasive. The above comments discussing why appellants are not persuasive are applied here as set forth above.

At page 46, appellant discusses what appellant perceives as the differences between the claims and the cited prior art and in particular to claim 53 with reference to the start signal. The comment that no reference teaches or suggests the claimed invention is noted but is erroneous as is the allegation of using the teaching of the application disclosure. The comment is noted and not well taken as no use of the present application disclosure is needed, used, implied, suggested, or inferred. For claim 53, there is no need in particular for any start codon since there is no recitation of same in the claim. Attention is directed to all of the above grounds of rejection over art.

Claim 53 is reproduced below for convenience of the Board as demonstrative of the fact that there is no *per se* recitation of nor need for a start codon requirement by or in the claim.

> 53. A method for detoxifying an organophosphorous compound comprising exposing said compound to recombinant bacterial organophosphorous acid anhydrase.

Here, for example McDaniel (BY) sets forth exposing the compounds to the enzyme (see page 2307 under phosphotriesterase assay) where parathion is hydrolyzed (i.e., detoxified) via an enzyme produced from a DNA inserted into a plasmid (see page 2307), i.e., a recombinant enzyme. Moreover, Wild *et al.* disclose exposing the organophosphorous acid anhydrase to organophosphorous compounds (see at least pages 629-630) as well as cloning and expression of DNA coding for organophosphate degrading enzymes from *P. diminuta* and a *Flavobacterium*. Thus, the claim is anticipated by the references and when combined with other references, makes all of the claims obvious Therefore, the assertion by appellant in the brief of using appellant's written description is erroneous and wrong.

As to the assertion of no reference teaches purifying the enzyme, the comments are noted but are not persuasive since there is no claim on appeal that contains the word "isolating" or "purifying" the enzyme or the DNA. This assertion by appellant is not commensurate in scope to the present claims which contain no such limitation and to read appellant's limitations into the claims is improper. See to read into the claims, limitations from the table is an incorrect reading of limitations into the claims - see *In re Zletz*, cited *supra*.

In the paragraph bridging pages 46-47, the brief asserts that no reference or combination of references teaches/suggests a start site or characterized the sequence necessary. The comments are not persuasive for the reasons discussed above in regard to claim 53 wherein appellant has stated that all of the claims stand or fall together - see brief page 11 and the above paragraphs as well as the stated grounds of rejection which set forth the fact that the claims are rejected without resort to appellant's disclosure. Thus, the comments are not persuasive and allegation of using appellant's disclosure is not well taken.

At page 47, the brief refers to seven limitations. As to the first point of few sequences for genes from soil bacteria, the comment is not persuasive since the issue is not whether or not other sequences were or were not known, rather it is the fact that the references cited in the grounds of rejection demonstrate that the DNA encoding the enzyme was known as is and was the enzyme that effected detoxifying organophosphorous compounds. As to the second point, the references cited in the grounds of rejection do not need nor require the prediction of a start site nor does, for example, claim 53 recite a start site, promoter, internal sequences, or terminators. Attention is directed to the above reproduction of claim 53. As to the third and fourth points of having a high G+C content, like point 2, it would argue reading into the claims (as for example claim 53) limitations that are not found in the claims. This is an incorrect interpretation by appellant of the appealed claims. The fifth point which argues the references teach away from the present invention is noted but for the above indicated reasons, the assertion in appellant's brief is not persuasive nor correct. The sixth point asserts that the references must lead one to make the combinations. This assertion in the present brief on appeal is unpersuasive because the stated grounds of rejection do indicate a reason for combining the references wherein Munnecke (AW) disclose (page 258) that the enzyme was needed, it would have been obvious to one of ordinary skill in the art to combine the disclosures of Munnecke (AW) and Munnecke (CD) with that of Wild *et al.* (AT) who disclose exposing the organophosphorous acid anhydrase to organophosphorous compounds (see at least pages 629-630) as well as cloning and expression of DNA coding for organophosphate degrading enzymes from *P. diminuta* and a *Flavobacterium* which enzymes would have been used in the disclosed process of degrading organophosphorous compounds set forth in both Munnecke references. Given the starting materials and teachings in the Wild *et al.* reference or the McDaniel *et al.* reference, it would have been obvious

that the ordinary skilled artisan would have obtained enzyme that was the same as that of the claims and which provides a source of the enzyme for use in the process set forth by both Munnecke references and Gottlieb who discloses that such enzymes can be used to detoxify gaseous phase organophosphorous compounds (see at least col 3). Moreover, it would have been obvious by logical deduction from the Gottlieb patent by one of ordinary skill in the art that organophosphorous degradation by the enzyme would have prevented contamination by pretreatment of an area with the enzyme which degrades the compound and that one such piece of protective equipment was a gas mask. Thus, one of ordinary skill in the art would clearly have recognized and used the combined references as all are directed to the same field of art, to the same invention, to the same subject matter, and to the solution of the same problem. Thus, the cited references also are properly combined and do teach and suggest the necessary elements for the claims which makes appellant's seventh and last point also unpersuasive.

In the boldface type at the bottom of page 47 and the paragraph bridging pages 47-48, the brief against asserts that there is no teaching or suggestion of expression at levels necessary to achieve successful purification. The comments are noted but are not persuasive. No claim on appeal contains, uses, needs, nor recites the utilization of a purified enzyme. Attention is directed to the above reproduction of claim 53. In the appendix of claims, no claim recites the presence nor use of a purified nor even a purified preparation of the enzyme. Thus, the assertions in the brief are not commensurate in scope to the present claims. As to the assertions regarding heterologous promoters the cited references, for example McDaniel *et al.* and Wild *et al.* disclose using plasmids with a *lac* and a *tac* promoter respectively nor does any claim require the presence or use of a heterologous promoter and none are recited in any claim on appeal.

In the first full paragraph of page 48, the brief asserts that no art suggests membrane associated protein could be expressed as an active protein. The comment is noted but not persuasive since Wild *et al.* at page 633 indicates that the membrane form of the enzyme has been kept frozen for months without appreciable loss of activity - i.e., one has a large quantity of the enzyme since it can be stored for months. Thus, the comments in the second paragraph of page 48 is not persuasive.

At page 48 to the end of the paragraph bridging pages 49-50, the brief discusses the level of ordinary skill in the art. It is noted that the decisions in *Standard Oil Co. v. American Cyanamid Co.*, *Ex parte Chicago Rawhide Manufacturing Co.*, *and In re Corkhill* have been cited. While it is noted that the *Standard Oil Co. v. American Cyanamid Co.* decision indicates that one of ordinary skill in the art is not one who innovates, it is not necessary to innovate to take a disclosed plasmid containing a given gene and to place that DNA into other plasmids and host cells as that technique has been used since at least the inception of genetic engineering technology. Moreover, and more recently, *In re Nilssen*, 7 USPQ2d 1500 (Fed Cir 1988), indicated that the hypothetical person of ordinary skill in the art is assumed to have knowledge of all prior art in the field of the inventor's endeavor, of prior art solutions for a common problem even if outside the field, and that for the purposes of combining references, those references need not explicitly suggest combining teachings, moreover, note the presently cited references in the grounds of rejection are a few of many references indicating the level of ordinary skill in the art. With regard to the motivation to combine (*Ex parte Chicago Rawhide Manufacturing Co.*), note the *In re Nilssen* decision and the fact that all of the cited references are drawn to the same field of endeavor as defined by the claims in the present application. In any event as previously indicated, in the rejections, the cited references disclose foreign genes are expressed using vectors and are widely accepted for expression of proteins of agricultural and medical importance such that one of ordinary skill in the art would have been motivated to use the plasmids microbial host cells because the heterologous products produced are biologically active and produce recombinant products very similar to the authentic proteins and because the vectors effect expression of prokaryotic (here the organophosphorous acid anhydrase DNA, an agriculturally important protein since it catalyzes the transformation of various organophosphorous pesticides) or eukaryotic genes to produce fused or non-fused recombinant proteins for the advantage of abundant expression of recombinant proteins. Thus, given what was known in the art, one of ordinary skill in the art would have found it obvious to combine the references with known art recognized techniques to have arrived at the invention, see *In re Donohue*, 226 USPQ 619 (CAFC 1985) which at page 621, like here, possession of the invention is effected when one of ordinary skill in the art (at least a PhD with some postdoctoral experience) combines the references disclosing the DNA coding for the OPD enzyme on a given plasmid, and which DNA has been cloned by insertion into alternative vectors where absent factual evidence to the contrary, any other alternative vector and host cell would have been an equivalent substitute within the

purview of one of ordinary skill in the art. Here, as indicated in the *In re Donohue* decision, that description in the art would have sufficed to permit one of ordinary skill in the art to have combined the publications' written description with the ordinary skilled artisans' own knowledge to so as to have arrived at the claimed invention. Thus, applicants' specification has not been used, implied or needed to reject the claims and the *Ex parte Chicago Rawhide Manufacturing Co.* decision is distinguished and no use of hindsight is implied, used, intended, or applied for which the *In re Corkill* decision is distinguished. Note that the application of the requisite art to reject the claims is not hindsight and such allegations are not well taken.

Starting at page 50, under secondary considerations, it is noted that the brief cites the *Custom Accessories, Inc. v. Jeffrey-Allan Industries, Inc.* decision as to long-felt unresolved need, commercial success, failure of others, and copying. The comments in the brief (pages 50-56) have been considered but are not persuasive for the reasons discussed below.

Appellant first discusses the aspect of long-felt but unresolved need and states that safe and effective elimination/protection against the toxic effects of organophosphorous compounds is well known and refers to two television broadcasts. The first of which is apparently the 13 minute segment of NBC Today (exhibit D) at pages 26-28. Appellant asserts this transcript demonstrates long-felt unresolved need, however, the transcript does not state this. The transcript does not even appear to indicate that detoxification is a need as for example Warren Stickle indicates (page 27) that "there's a long history of usage of lawn care chemicals without any adverse effects to human health", i.e., just the opposite of long-felt unresolved need since it points to no the absence of any problem. The further discussion by Dr. Moses points to a health hazard to be sure, however, there is no indication with regard to necessity of or for a process for detoxification. The transcript refers to better labeling of the products - this is not a process of detoxification. In fact, at pages 29-31 the transcript asserts that one does not need to use chemicals in the first place. Thus, the first transcript (exhibit D) is not persuasive.

In the second full paragraph of page 51, the brief refers to exhibits F through K as a collection of pesticide fact sheets that are asserted to show concern regarding compounds such as ethyl parathion, Diazinon, fenitrothion, coumaphos, acephate, and malathion. The fact sheets do not

indicate any long-felt unresolved need nor even refer to the presently claimed process at all. Thus, reference and/or reliance upon exhibits F to K are not persuasive.

In the second transcript ABC World News Tonight (exhibit C) the last full paragraph of page 51 refers to a case of Diazinon poisoning, however, it does not demonstrate nor refer to any long-felt unresolved need nor is there any reference to any detoxification process nor any reference to the presently claimed process. Thus, the comments are not persuasive.

In the first paragraph of page 52, appellant refers to a letter (exhibit N) to Ms. A. Levy and a letter to Dr. D. R. Eger (exhibit L and M). As to exhibit N, the letter does not indicate any long felt need. In fact, it is apparent from the letter that R. B. Tabakin is not even sure what the process is that is available (nor does the letter indicate what technology is being referred to) nor does the letter appear to even refer to the presently claimed process. Exhibit L like the others, does not demonstrate any long-felt need and research, exhibits L and M, are definitely a not long-felt need.

In the paragraph bridging pages 52-53, it is noted that the brief also refers to exhibit A, the article in the New York Times. The comments are not persuasive of any long-felt need nor does the article even refer to the presently claimed process as a long-felt unresolved need. Funding for research is not and does not demonstrate any long-felt unresolved need nor demonstrate any commercial success in the face of an admitted in the brief, nonexistent market. The fact that the U.S. Army has a stockpile of the pesticide and has defined a route of eliminating the pesticide by burning same does not establish a need for the DNA, the vectors, host cells, or a process utilizing recombinant DNA technology to use the enzyme. Thus, the comments are not persuasive nor demonstrate any nexus to commercial success. Research is not a commercial use of the process as presently claimed.

Under the heading of (asserted) commercial success, the brief refers to a pilot scale process but has provided no factual documents for same. The same exhibit M is referred to but no facts are presented as to what is part of the development phase and what was to be done and research does not demonstrate commercial success. This does not translate into a showing of commercial success especially where as admitted in the response, appellant have no data showing the process to be

effective in any commercial endeavor and in the last full paragraph of page 53 admit that there is effectively, none. Appellant admits the market is undeveloped. Thus, contrary to appellant's comments, there is no nexus established as for example, there is no apparent factual evidence of licensing (see for example *Stratoflex, Inc. v. Aeroquip Corporation*, 218 USPQ 871 (Fed Cir 1983) nor are there any facts of record as to any recognition and acceptance by competitors nor is there any delineation of others in the industry (where appellant would admit of no industry at all since appellant admits it is not developed).

It is noted that appellant refers to employment of Serdar at Amgen and infers that Amgen would not provide funding for projects with no commercial success. So noted, however, it is unpersuasive. There are no declaratory facts to this effect from either Serdar or Amgen. Moreover, the inference regarding Amgen and commercial success appellant puts forward is not reflected in the 1989 Serdar *et al.* reference. Insofar as appellant asserts the work carried out by Serdar *et al.* was done despite the cited references, Serdar *et al.* are not the present inventors and there is no declaratory statement to that effect from Serdar. Thus, the comments in appellant's brief are not persuasive.

The comments in the response at page 54 assert copying by others and cites *Specialty Composites v. Cabot Corp.* decision. Neither the Serdar *et al.* nor the Mulbry *et al.* references indicate that they copied from appellant. Here, the Mulbry *et al.* reference indicates that the strains were previously described cites reference 10 and 17 from the Mulbry *et al.* reference where reference 10 is a reference by Mulbry and Karns 1989 (Appl. Environ. Microbiol. 55: 289-293) and Sethunathan *et al.* (neither of which is appellant). As to the Serdar *et al.* 189 [sic] reference it appears to be the 1989 reference. That 1989 Serdar *et al.* reference (page 115) in the left column refers to the authors' own early work. The Serdar *et al.* authors are not appellant and can hardly be said to have copied from appellant when the Serdar *et al.* authors refer to their own work. In view of the foregoing, appellant's assertion of copying by others is not persuasive. Thus, the *Specialty Composites v. Cabot Corp.* decision is distinguished.

Appellants brief at page 55-56 asserts the failure of others, however, that is not apparent from the successful examples presented in the cited prior art. The comments in the brief do not establish a competitive commercial market nor demonstrate commercial success, do not demonstrate praise of independent commentators nor any performance benefits of the DNA, the vectors, and the host cells. Note that one cannot infringe a nonexistent patent nor do the facts of record presented in the response establish same, thus, the *S.C. Johnson & Son, Inc. v. Carter-Wallace, Inc.* decision cited by in the brief is distinguished. At page 56 it is noted that the brief asserts that others failed to identify the start signal, the sequence of the gene, express the protein from the gene, and transform eukaryotic cells with the gene. The comments are noted, however, they are not persuasive because without identification of the start signal, others as set forth in the cited grounds of rejection were able to effect expression of the DNA to produce the enzyme (i.e., the start signal was present in the constructs used) and where the cited references contain the DNA encoding the enzyme, the DNA contains the sequence (sequencing a DNA does not alter the DNA *per se* nor its sequence). Moreover, the cited references disclose that expression of the DNA was obtained in the form of the enzyme encoded by that DNA and thus, appellant's comments in the brief are not persuasive as to inability to produce the enzyme. The comments also assert failure to transform eukaryotic cells. The comment is noted but is not persuasive as it is known in 1988 to use eukaryotic cells to express heterologous DNA and Munnecke (AW) at page 262 indicates that eukaryotic organisms are already known to metabolize malathion. *Aspergillus* is a eukaryote and the DNA contained therein is recombinant. Thus, appellant's comments regarding asserted failure is not substantiated by factual scientific data.

In view of the foregoing, appellant's assertion of compelling nonobviousness is unpersuasive. The conclusions presented in the last full paragraph of appellant's brief at page 56 is noted but is also unpersuasive for the reasons set forth above. Here, each refrence in the rejections under 35 U.S.C. 102 and the combined references used in the rejections under 35 U.S.C. 103 disclose a method for detoxifying an organophosphorous compound comprising exposing said compound to recombinant bacterial organophosphorous acid anhydrase. For the above indicated reasons, the above rejections should be affirmed.

Respectfully,

Christopher S. Low

Christopher Low
30 January 1996

5

CHRISTOPHER S. F. LOW
PRIMARY EXAMINER
GROUP 1800

Attached is the marked copy of
Appellant's exhibit B

COMPARISON OF opd SEQUENCE DISCLOSED IN PATENT APPLICATION SERIAL NO. 07/344,258 TO PUBLISHED SEQUENCES

1 = Patent Application
2 = McDaniel et al. (1988)
3 = Harper et al. (1988)
4 = Serdar et al. (1989)
5 = Mulby & Karns (1989)
6 = Corrected Sequence

```
                      x  b  q
1  C T G C A G        -  -  -  C C T G A C T C G G C A C C A G T C G C T   30
2                     -  -  -
3                     -  -  -
4                     G  T  C
5                     -  -  -
6                     -  -  -

1  G C A A G C A G A G T C G T A A G C A A T C G C A A G G G G              60
2
3
4
5
6
```

opd coding Sequence

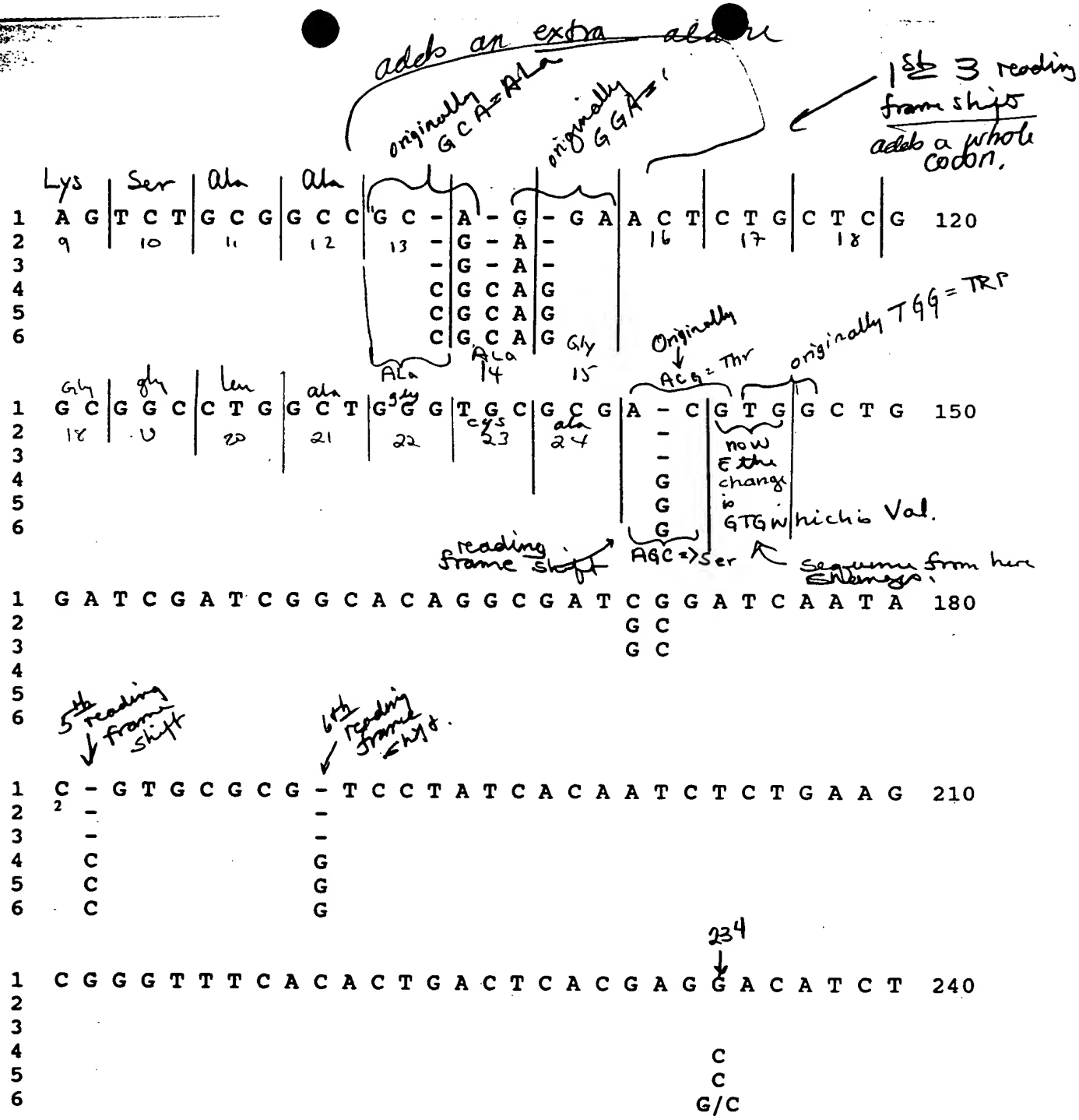
```
                        Met      Gln      Thr      Arg      Arg      Val      Val      Leu
1  G C A G C  A T G¹  C A A  A C G  A G A  A G G  G T T  G T G  C T C  A   90
2             1        2        3        4        5        6        7        8
3
4
5
6
```

---

¹ consensus start codon

[ ]= a blank space means identity (homology) with the sequence in the patent application

[-]= a hyphen means a base is missing in the sequence in which hyphen occurs but which base occurs in another sequence

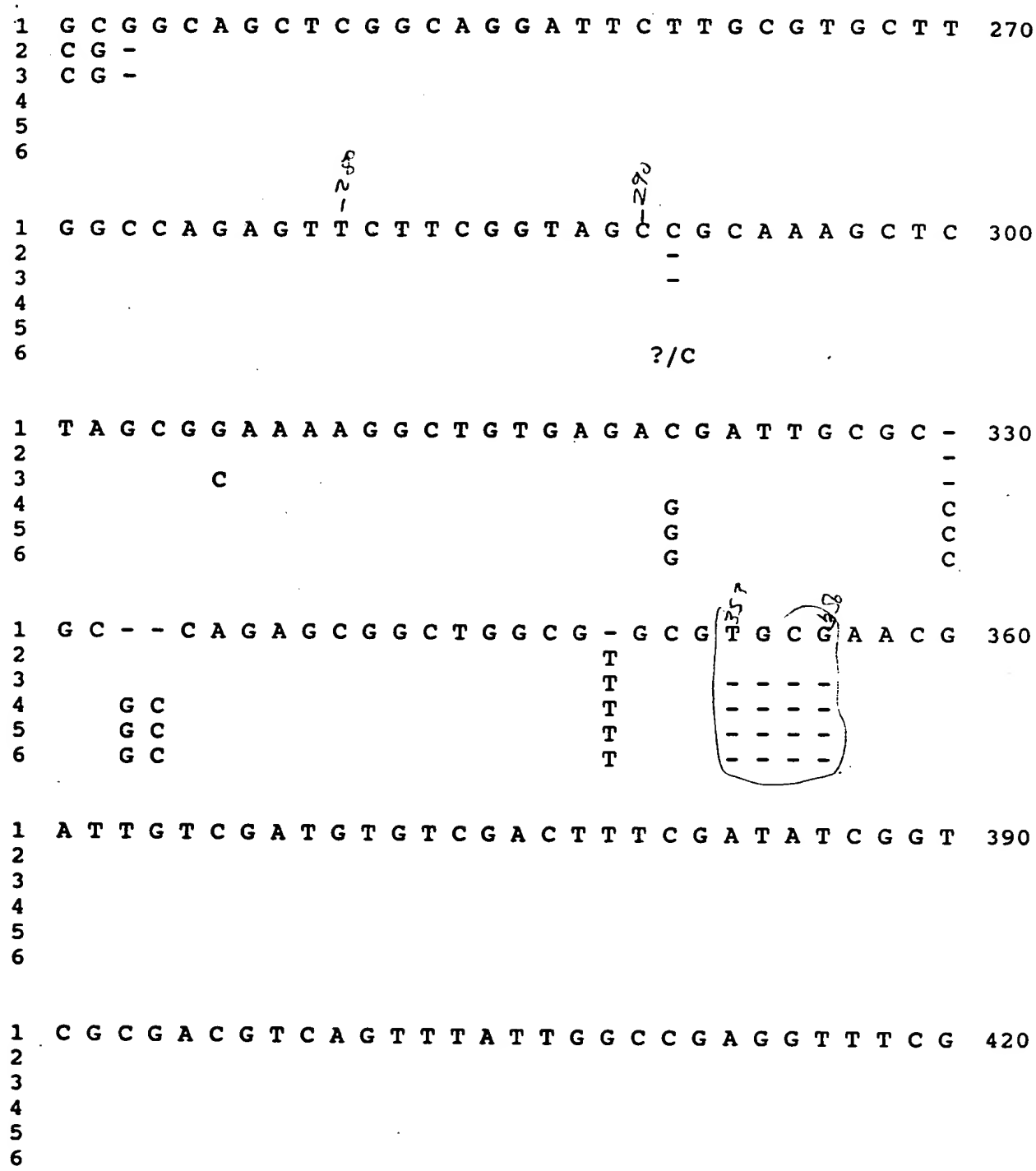


---

[2] Mulby and Karns (1989) cite in their Fig. 5 that Harper et al. (1989) has a "T" at this position. That is incorrect. Harper et al. show a "C" at this position.

[ ]= a blank space means identity (homology) with the sequence in the patent application

[-]= a hyphen means a base is missing in the sequence in which hyphen occurs but which base occurs in another sequence



[ ]= a blank space means identity (homology) with the sequence in the patent application

[-]= a hyphen means a base is missing in the sequence in which hyphen occurs but which base occurs in another sequence

```
1  C G G G C T G C C G A C G T T C A T A T C G T G G C G G C G  450
2                                          -
3                                          -
4
5
6


1  A C C G G C T T G T G G T T C G A C C C G C C A C T T T C G  480
2
3
4
5
6


1  A T G C G A T T G A G G T A T G T A G A G G A A C T C A C A  510
2
3
4                        A G
5                        A G
6


1  C - A G T T C T T C C T G C G - T G A G A T T C A A T A T G  540
2    T                       G
3    T                       G
4
5
6


1  G C A T C G A A G - A C A C C G G A A T T A G G G C G G G C  570
2                    T
3                    T
4
5
6


1  A T T A T C A A G G T C G C G A C C A C A G G C A A G G C G  600
2
3
4
5
6
```

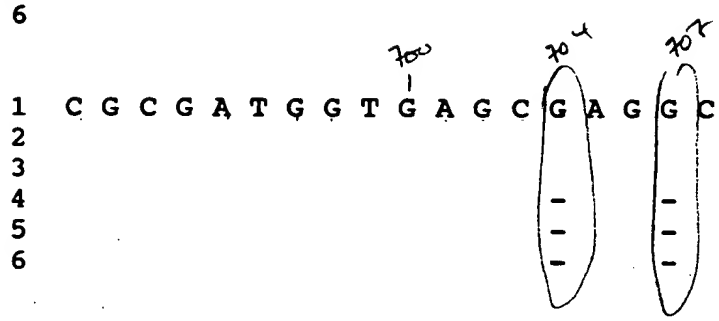
[ ]= a blank space means identity (homology) with the sequence in the patent application

[-]= a hyphen means a base is missing in the sequence in which hyphen occurs but which base occurs in another sequence

```
1  A C C C C C T T T C A G G A G T T A G T G T T A A A G G C G  630
2
3
4
5
6


1  G C C G C C C G G G C C A G C T T G G C C A C C G G T G T T  660
2
3
4
5
6


1  C C G G T A A C C A C T C A C A C G G C A G C A A G T C A G  690
2
3
4
5
6
```




```
1  C G C G A T G G T G A G C G A G G C A G G C C G C C A T T T  720
2
3
4                          -   -
5                          -   -
6                          -   -


1  T T G A G T C C G A A G - C T T G A G C C G - T C A C G G G  750
2                        -                 -
3                        -                 -
4                        G                 C
5                        G                 C[3]
6                        G                 C
```

---

[3] Incorrectly left out of Mulbry and Karns as a difference in Fig. 5.

[ ]= a blank space means identity (homology) with the sequence in the patent application

[-]= a hyphen means a base is missing in the sequence in which hyphen occurs but which base occurs in another sequence

```
1  T T T G T A T T G G T C A C A G C G A T G A T A C T G A C G  780
2
3
4
5
6


1  A T T T G A G C T A T C T C A C C G C C C T - G C T G - - C  810
2                                           -       - -
3                                           -       - -
4                                           C       C G
5                                           C       C G
6                                           C       C G


1  G C G G A T A C C T C A T C G G T C T A G A C C A C A T C C  840
2
3
4
5
6


1  C G C A C A G T G C G A T T G G T C T A G A A G A T A A T G  870
2
3
4
5
6


1  C G A G T G C A T C A C C G C T C C T G G G C A T C C G T T  900
2
3
4                       G   C
5                       G   C
6                       G   C


1  C G T G G C A A A C A C G G G C T C T C T T G A T C A A G G  930
2
3
4
5
6
```

[ ]= a blank space means identity (homology) with the sequence in the patent application

[-]= a hyphen means a base is missing in the sequence in which hyphen occurs but which base occurs in another sequence

```
1  C G C T C A T C G A C C A A G G C T A C A T G A A A C A A A  960
2
3
4
5
6


1  T C C T C G T T T C G A A T G A C T G G C T G T T C G G G T  990
2
3
4
5
6


1  T T T C G A G C T A T G T C A C C A A C A T C A T G G A C G  1020
2
3
4
5
6


1  T G A T G G A T C G C G T G A A C C C C G A C G G G A T G G  1050
2
3
4
5
6


1  C C T T C A T T C C A C T G A[4] G A G T G A T C C C A T T C -  1080
2                      -
3                      -
4                                                          C
5                                                          C
6
```

[4] Stop codon postulated by patent application.

[ ]= a blank space means identity (homology) with the sequence in the patent application

[-]= a hyphen means a base is missing in the sequence in which hyphen occurs but which base occurs in another sequence

```
1  T A C G A G A G A A G G G C G T C C C A C A G G A A A C G C  1110
2
3
4
5
6


1  T G C C A G G C A T C A C T G T G A C T A A C C C G G C G C  1140
2      G
3      G
4      G
5      G
6      G


1  G G T T C T G T G T C A C C G A C - T T G C - - - C G T G C  1170
2                                    -       - - -
3                                    -       - - -
4                  -                 C       G G G         -
5                  -                 C       G G G         -
6                  -                 C       G ?5?         ?


1  A T G6 A C G C C A T C T G G A T C C T T C C A C G C A G C G  1200
2
3
4                                                  G C
5                                                  G C
6                                                  ? ?


1  G C C A C T A T T C C C C G T C A A G A T A C C G A A C G A  1230
2
3
4  C G
5
6  ? ?
```

---

[5] Inventors have not re-sequenced the DNA 3' of this point since it most likely is not included within the open reading frame.

[6] Stop codon postulated by Mulbry and Karns/Serdar et al.

[ ]= a blank space means identity (homology) with the sequence in the patent application

[-]= a hyphen means a base is missing in the sequence in which hyphen occurs but which base occurs in another sequence

```
                                    -1240                   1244  -1251
1  T G A A G T C G C G C A T C G A T C G A T A G G C A T C T T  1260
2                                   - - - -
3
4
5
6                                   ? ? ? ?

                        1256
1  C A A T G T G A T C A G G G C T G C C A C C T C C A A A G C  1290
2
3
4
5           T 7
6           ? ?

1  C G G T G G C C A C C C C T G T C G A T A G T C T T G A G G  1320
2
3
4      -
5      -
6

1  G A C G G T A G C G A C G A C C G T G C T T T T C G T G A A  1350
2
3
4  -       -       G   C A C                         - -
5  -       -       G   C A C                         - -
6

1  C T G C A G  1356
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[7] Mulbry and Karns incorrectly show Harper et al. places a "C" here.

[ ]= a blank space means identity (homology) with the sequence in the patent application

[-]= a hyphen means a base is missing in the sequence in which hyphen occurs but which base occurs in another sequence

Notice of References Cited

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08/252,384	McDaniel et al.		
Examiner		Group Art Unit	Page 1 of 1
Christopher S. F. Low		1804	

U.S. PATENT DOCUMENTS

		DOCUMENT NO.	DATE	NAME	CLASS	SUBCLASS
	A					
	B					
	C					
	D					
	E					
	F					
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	J					
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FOREIGN PATENT DOCUMENTS

		DOCUMENT NO.	DATE	COUNTRY	NAME	CLASS	SUBCLASS
	N						
	O						
	P						
	Q						
	R						
	S						
	T						

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Nov 30 Jan 1994

[36] High-Sensitivity Sequencing with a Gas-Phase Sequenator

By MICHAEL W. HUNKAPILLER, RODNEY M. HEWICK, WILLIAM J. DREYER, and LEROY E. HOOD

Since its introduction in 1967, automated Edman degradation has been the most widely used method for the direct determination of the primary structure of proteins.[1] Both the liquid-phase, or spinning-cup, sequenator developed by Edman[1] and the solid-phase sequenator developed by Laursen[2] have been available commercially for a decade, the former from Beckman Instruments (Palo Alto, California) and the latter from Sequemat (Watertown, Massachusetts) and LKB (Rockville, Maryland). Each has advantages for particular sequencing applications. The spinning-cup instruments offer simplicity of sample application and, with the use of Polybrene,[3] can be used to sequence both proteins and peptides. However, they were designed for analysis of what are today rather large quantities of sample (a few hundred nanomoles) and require large volumes of reagents that are difficult to obtain in sufficient purity for microsequencing applications. The solid-phase sequenators were designed for handling much less sample and can be used for both proteins and peptides. However, because they require covalent attachment of the sample to a solid support, they require an initial chemical treatment of the sample that often results in substantial loss of material even before the sequencing experiment itself begins.

A new type of sequenator, one that employs gas-phase reagents instead of liquid-phase reagents at critical points in the Edman degradation, has been described.[4] This gas-phase sequenator offers the simplicity of sample loading of the spinning-cup sequenators, the miniaturization of the solid-phase instruments, the versatility of sequencing both proteins and peptides, and the use of reagents that are easily purified. It provides superior performance, in terms of repetitive cycle yield and length of sequence obtainable, to both spinning-cup and solid-phase methodology when large amounts (10–100 nmol) of sample are available. Moreover, it can be used to analyze proteins and peptides with as little as 5 and 50

[1] P. Edman and G. Begg, *Eur. J. Biochem.* **1**, 80 (1967).
[2] R. A. Laursen, *Eur. J. Biochem.* **20**, 89 (1971).
[3] G. E. Tarr, J. F. Beecher, M. Bell, and D. J. McKean, *Anal. Biochem.* **84**, 622 (1978).
[4] R. M. Hewick, M. W. Hunkapiller, L. E. Hood, and W. J. Dreyer, *J. Biol. Chem.* **256**, 7990 (1981).

METHODS IN ENZYMOLOGY, VOL. 91

ISBN 0-12-181991-4

pmol, respectively, amounts below the capability of the other types of sequenators unless the sample contains intrinsic radiolabel. In the following report, we describe the design and operation principles of this gas-phase sequenator.

Reagent Preparation

Reagents and solvents used in automated Edman degradation must undergo rigorous purification to eliminate impurities that would otherwise interfere either with the Edman chemistry itself or with subsequent analysis of phenylthiohydantoin (>PhNCS) amino acids. Satisfactory purification methods for the sequenator chemicals are described below; chemicals of appropriate quality are also available from Applied Biosystems (Foster City, California). The chemicals, once purified, are generally stable for at least 6 months if they are protected against new contamination or breakdown. This requires storage in the dark under argon in Pyrex vessels sealed with Teflon-lined enclosures. When fresh supplies of the chemicals are loaded into the sequenator reservoirs and these are attached to the sequenator, the air space above the liquid level must be purged with argon using the reservoir vent and pressurize system in order to prevent atmospheric contamination.

Reagent 1. The coupling reagent is a 17% phenylisothiocyanate solution in *n*-heptane. The phenylisothiocyanate is purchased in 2.5-ml sealed vials from Beckman Instruments and used as is or redistilled under vacuum at 0.2 mm Hg (bp 40°). Heptane is obtained from Burdick and Jackson (Muskegon, Illinois). It is stirred for 1 hr with alumina (Activity I, Woelm Pharma, Federal Republic of Germany), filtered through a fine-frit sintered-glass funnel, and distilled under argon in a twisted Teflon spinning band column (Part Nos. 40T and 450-6, B/R Instruments, Pasadena, Maryland). The reagent solution can be stored in a desiccator (anhydrous $CaSO_4$) for at least 2 months without decomposition.

Reagent 2. The coupling buffer is a 25% trimethylamine solution in water. Anhydrous trimethylamine (Eastman Organic Chemicals, Rochester, New York) is purified by refluxing with phthalic anhydride (Matheson, Coleman, and Bell, Norwood, Ohio) at 5° for 2 hr and distillation through a Pyrex column filled with phthalic anhydride into a receiving flask cooled in a dry ice–acetone bath. Water is deionized and distilled in an automated glass still (Megapure 6, Corning Glass Works, Corning, New York). The reagent solution is stored at −20° in a 250-ml centrifuge bottle (Part No. 1261-200, Corning), sealed with a Kalrez O-ring (Part No. AS-568A-214-1050, E. I. DuPont de Nemours and Company, Wilmington, Delaware), and Teflon-lined screw cap. When the reagent solution is

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's obtained from Burdick and
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lany), filtered through a fine-frit
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ored in a desiccator (anhydrous
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5% trimethylamine solution in
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ith phthalic anhydride (Mathe-
) at 5° for 2 hr and distillation
alic anhydride into a receiving
ater is deionized and distilled in
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20° in a 250-ml centrifuge bottle
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When the reagent solution is

loaded into the sequenator and used, the more volatile trimethylamine will tend to bubble off leaving behind the water containing a decreasing concentration of base. Therefore, the used reagent should be replaced with fresh reagent when the reagent volume in the reservoir decreases to below 85% of the initial amount.

Reagent 3. The cleavage reagent is anhydrous trifluoroacetic acid. The acid (Eastman) is first distilled under dry argon from Cr_2O_3 in a 30-cm Widmer column. The fraction boiling at 72° is stirred with alumina (Woelm), decanted from the alumina, and redistilled under argon from dithiothreitol (50 mg per 100 ml of acid, Ultrol grade, Calbiochem, La Jolla, California). Dithiothreitol (10 mg 100 ml of acid) is added to the purified reagent just before the acid is loaded into the sequenator.

Reagent 4. The conversion reagent is a 1 *N* methanolic HCl solution prepared according to Tarr.[5] Acetyl chloride (J. T. Baker Chemical Company, Phillipsburg, New Jersey) is distilled (bp 52°) in a 30-cm Widmer column. Methanol (HPLC grade, Baker) is purified by treatment with activated charcoal at 40°, filtration through a fine-frit sintered-glass funnel, and distillation under argon in the spinning band column. In order to make the reagent, stir the methanol (95 ml) in an ice bath and add the acetyl chloride (8 ml) dropwise.

Solvent 1. Benzene (HPLC grade, Baker) is stirred for 1 hr with alumina, filtered through a fine-frit sintered-glass funnel, and distilled under argon in the spinning band still. Dithiothreitol (10 mg per liter of benzene) is added to the solvent just before it is loaded into the sequenator. Dissolution of the dithiothreitol is effected by immersion of the solvent reservoir in an ultrasonic water bath for a few seconds.

Solvent 2. Ethyl acetate (HPLC grade, Baker) is stirred for 15 min with activated charcoal and alumina at 40°, allowed to cool to room temperature with continued stirring, and filtered through a fine-frit sintered-glass funnel. It is distilled under argon from dithiothreitol (100 mg per liter of EtOAc) in the spinning band column. Acetic acid (0.5 ml per liter of EtOAc, aldehyde-free grade, Baker) and dithiothreitol (20 mg per liter of EtOAc) are added to the solvent just before it is loaded into the sequenator.

Solvent 3. 1-Chlorobutane (Burdick and Jackson) is stirred for 1 hr with alumina, filtered through a fine-frit sintered-glass funnel, and distilled under argon in the spinning-band still. Dithiothreitol (10 mg per liter of BuCl) is added to the solvent just before it is loaded into the sequenator. Dissolution of the dithiothreitol is effected by immersion of the solvent reservoir in an ultrasonic water bath for a few seconds.

[5] G. E. Tarr, *Anal. Biochem.* 63, 361 (1975).

Solvent 4. Methanol is prepared as described above for reagent 4. Dithiothreitol (10 mg per liter of MeOH) is added to the solvent just before it is loaded into the sequenator.

Sample Preparation

There are three prime considerations for preparation of protein or peptide for sequence analysis by the sequenator. These are (*a*) absence of nonvolatile (or 1-chlorobutane-insoluble) buffers or salts; (*b*) use of high-purity reagents; and (*c*) ability to concentrate the sample solution to a small volume (25–30 μl). Suitable sample loading solutions include distilled water and aqueous solutions of acetic acid, trifluoroacetic acid, ammonia, ammonium bicarbonate, ammonium acetate, or sodium dodecyl sulfate (SDS) (electrophoresis grade, Bio-Rad, Richmond, California). Reagent (or higher) grade chemicals must be used in order to prevent amino-terminal blockage of proteins. Formic acid, formate salts, and pyridinium salts are not recommended because they are frequently contaminated with amino-reactive impurities. A dilute (0.1%) solution of SDS is a convenient sample loading solution because the detergent is a good protein solubilizer and minimizes adsorption of protein to glass or plastic. The latter characteristic is particularly important when dilute solutions of small quantities of proteins must be handled. The SDS also promotes redissolution of proteins concentrated to dryness from volumes of solution too large for convenient loading into the sequenator. A convenient and rapid method of concentration is to place the sample solution in a 1.5-ml plastic conical centrifuge tube and evaporate the liquid with a gentle stream of nitrogen directed onto its upper surface.

Sequenator Design

The design of the sequenator has been described in detail by Hewick *et al.*[4] and is depicted schematically in Fig. 1. A sequenator based upon this design is currently available from Applied Biosystems. Argon (or nitrogen) is delivered through a series of filters and regulators to pressurize the reagent and solvent reservoirs. The argon pressure is used to force liquid or reagent gas from the reservoirs through a manifold of zero-holdup, solenoid-actuated, diaphragm valves to the reaction cartridge. Other valve manifolds of the same type direct effluent from the cartridge to waste, vacuum, or a second reaction vessel where the anilinothiazolinones removed from the protein in the cartridge are converted to phenylthiohydantoins. The latter are transferred with solvent to 300-μl tubes in a small fraction collector, where they are stored pending analysis. The valves

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ding solutions include dis-
acid, trifluoroacetic acid,
acetate, or sodium dodecyl
d, Richmond, California).
used in order to prevent
acid, formate salts, and
e they are frequently con-
ute (0.1%) solution of SDS
se the detergent is a good
protein to glass or plastic.
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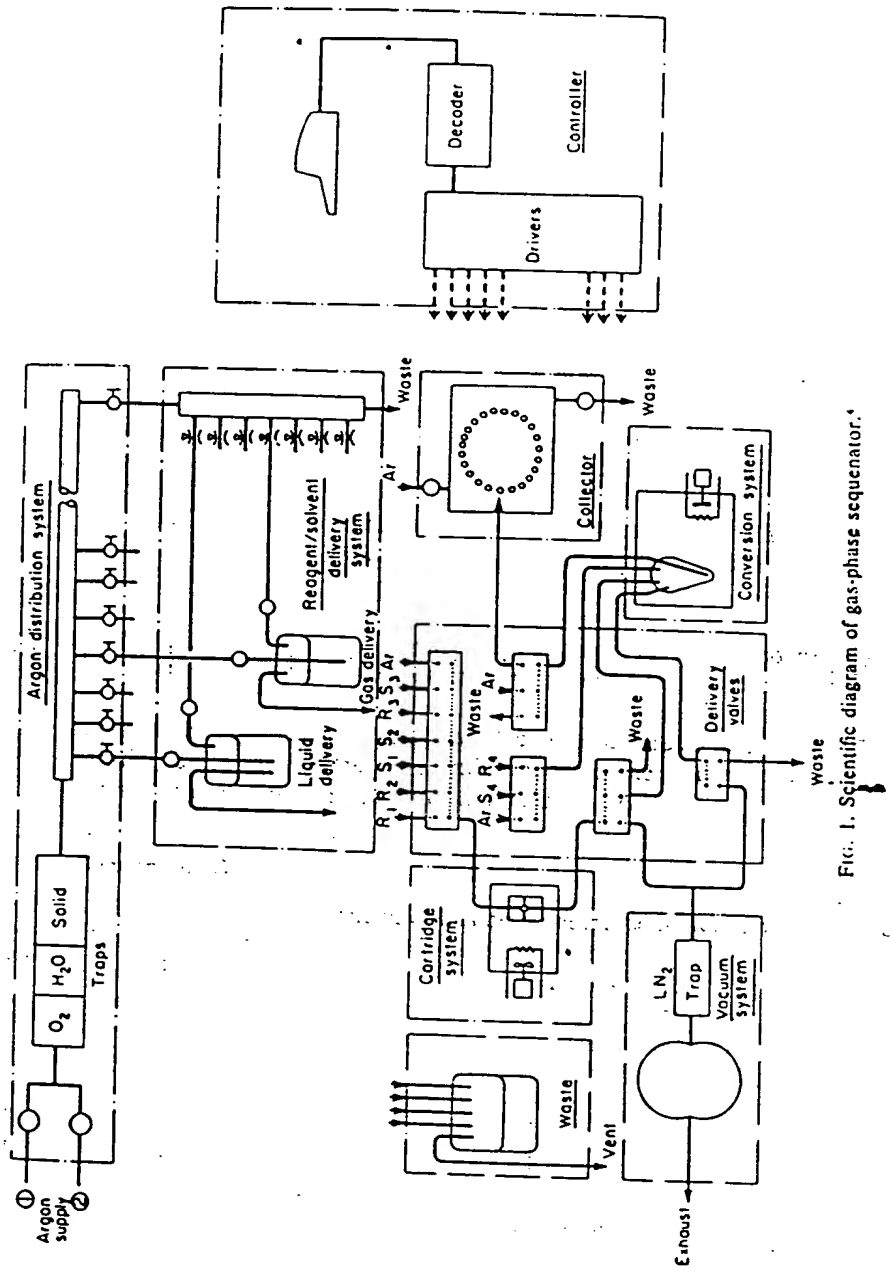

FIG. 1. Scientific diagram of gas-phase sequenator.[4]

controlling all reagent, solvent, and argon transfers can be operated manually or automatically by a solid-state electronic programmer. The only materials in contact with reagent and solvent gas or liquid are Pyrex glass, Teflon, Kel-F, Kalrez, and stainless steel (vacuum train and reservoir vent manifold only).

The vacuum effluent from the reaction vessels passes through a stainless steel or Pyrex trap immersed in liquid nitrogen before it reaches the pump. Other liquid wastes or gaseous exhausts are passed into a Pyrex waste bottle vented to an exhaust hood in the laboratory housing the sequenator. Water (150–200 ml) is added to the waste bottle after each time the liquid waste accumulation is dumped. The water prevents salt crystal formation in the lines leading into and out of the waste bottle. The vacuum trap must be emptied every 2–4 weeks, depending on the size of the trap, and the vacuum pump oil should be changed every 4–6 weeks.

The protein or peptide is immobilized along with Polybrene by adsorption onto a glass fiber disk (GF/C Glass Microfibre Filter, Whatman Ltd., England). The glass fiber disk is contained in a reaction cartridge constructed from two cylinders of Pyrex glass rod (1-inch diameter × 1-inch length) with finely ground, vacuum-flat surfaces at both ends (Wilmad Glass Company, Buena, New Jersey). Each piece of glass is machined ultrasonically (L. C. Miller Company, Monterey Park, California) so that it has a 0.5-mm-diameter central capillary flared out to a 1.2-cm-diameter conical recess at one end. The recessed ends of the two glass cylinders are clamped together in a metal cylinder to form a central chamber. A porous, fibrous Teflon disk (Zitex filter membrane, extra-coarse grade,

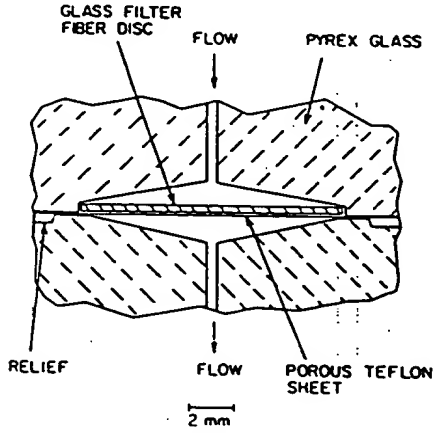

FIG. 2. Enlarged detail of reaction chamber.[4]

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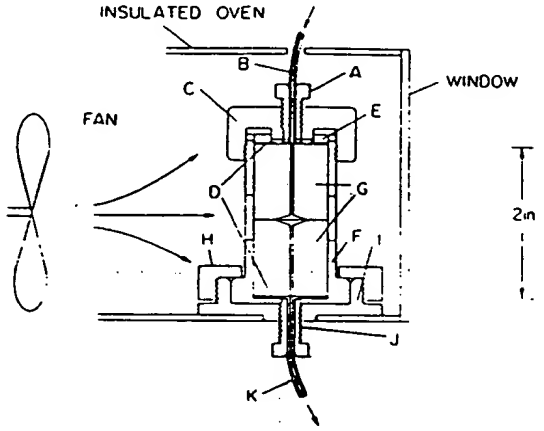


FIG. 3. Cartridge assembly. A, inlet aluminum fitting; B, inlet Teflon tubing; C, aluminum cap; D, Teflon washer; E, keyed aluminum washer; F, 304 stainless steel cartridge body; G, Pyrex glass rod; H, aluminum locking ring; I, aluminum mounting base; J, outlet aluminum fitting; K, outlet Teflon tubing.[4]

Chemplast, Wayne, New Jersey) is crushed between the abutting glass surfaces to effect a vacuum-tight seal and provide a physical support for the glass fiber disk. An enlarged detail of this arrangement is shown in Fig. 2.

A diagram of the entire reaction cartridge is shown in Fig. 3. The glass capillary openings at the ends of the reaction cartridge are connected to flanged Teflon tubing (0.3 mm i.d.) held against the glass surfaces by aluminum fittings. Access to the cartridge interior is achieved by loosening the upper tubing fitting, unscrewing the aluminum retaining cap, and removing the keyed aluminum washer, upper Teflon washer, and upper glass cylinder. Used Teflon and glass fiber disks can be removed and replaced with new ones and the cartridge can be reassembled by reversing the order of the disassembly steps. The keying of the aluminum washer into the stainless steel cylinder prevents rotation of the glass cylinders relative to each other or to the rest of the cartridge assembly when the aluminum retaining cap is screwed into place.

The sequenator is equipped with a second reaction chamber that allows automated conversion of the amino acid anilinothiazolinones extracted from the cartridge to >PhNCS amino acids. The apparatus (Fig. 4) is adapted from that described by Wittmann-Liebold *et al.*[6] It consists of a conical, Pyrex vessel with connections to four capillary feed or exit lines.

[6] B. Wittmann-Liebold, H. Graffunder, and H. Kohls, *Anal. Biochem.* 75, 621 (1976).

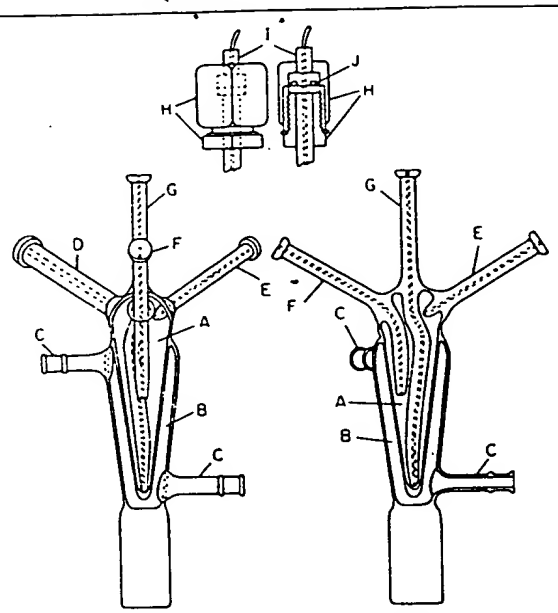


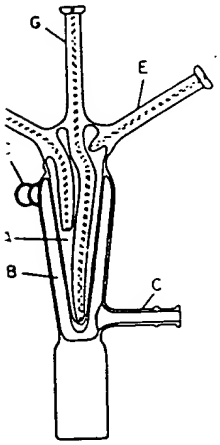
FIG. 4. Phenylthiohydantoin amino acid conversion flask. A, Pyrex vessel; B, water jacket; C, water bath port; D, vacuum/waste port; E, reagent/solvent delivery port; F, 1-chlorobutane extract delivery port; G, argon delivery/waste/fraction collector transfer port; H, nylon compression fitting; I, Teflon tubing fitting; J, O-ring groove.

The four lines provide (*a*) delivery of anilinothiazolinone solution from the cartridge; (*b*) delivery of conversion reagent, extraction solvent, and argon; (*c*) venting to waste line or evacuation by vacuum pump; and (*d*) delivery of argon into the flask, transfer of >PhNCS amino acid solution to the fraction collector, or transfer of liquid from the flask to the waste bottle. The delivery and removal functions are controlled by a series of the Teflon diaphragm valves described above. Tubing connections and seals are made by tightening interfitting, screw-thread connectors together to compress a Kalrez O-ring between a Teflon plug and a radial flange on the end of each of the capillary glass tubes protruding from the flask. The small internal volume (2 ml) of the flask and the sharp taper near the flask's lower tip allow efficient removal of >PhNCS amino acids with approximately 250 μl of solvent.

Sequenator Operation

The sequenator program is listed in Table I. The cartridge and flask programs run concurrently and are synchronized to prevent simultaneous

Step	
1	Pressu
2	Pressu vent
3	R3 del
4	Pressu
5	Vacuu
6	Pressu
7	Vacuu
8	Pressu
9	Pressu vent
10	S3 deli
11	S3 deli
12	S3 extr
13	S3 colle
14	S3 deli
15	S3 extr
16	S3 colle
17	S3 deliv
18	S3 extra
19	S3 colle
20	S3 deliv
21	S3 extra
22	S3 colle
23	Delay
24	Vacuum
25	Pressuri
26	Vacuum
27	Pressuri
28	Pressuri vent
29	R3 delive
30	R3 delive
31	R3 delive
32	R3 delive
33	R3 delive
34	R3 delive
35	Pressuriz
36	Vacuum
37	Pressuriz
38	Vacuum
39	Delay



ersion flask. A, Pyrex vessel; B, water rt; E, reagent/solvent delivery port; F, lelivery/waste/fraction collector transfer ; fitting; J, O-ring groove.

inothiazolinone solution from the gent, extraction solvent, and ar- ation by vacuum pump; and (*d*) of >PhNCS amino acid solution iquid from the flask to the waste ons are controlled by a series of above. Tubing connections and screw-thread connectors together Teflon plug and a radial flange on es protruding from the flask. The sk and the sharp taper near the al of >PhNCS amino acids with

Table I. The cartridge and flask chronized to prevent simultaneous

TABLE I
SEQUENATOR PROGRAM

Step	Cartridge functions	Flask functions	Step time (sec)	Volume (ml)
1	Pressurize	Pressurize	2	—
2	Pressurize, vent; R3 pressurize, vent	Vacuum	6	—
3	R3 deliver	Vacuum	400	5 cm³/min (gas)
4	Pressurize, vent	—	20	—
5	Vacuum	—	40	—
6	Pressurize	—	2	—
7	Vacuum	—	50	—
8	Pressurize	Pressurize	2	—
9	Pressurize, vent; S3 pressurize, vent	Pressurize, vent; S4 pressurize, vent	6	—
10	S3 deliver, collect	S4 deliver	8	0.04 (S3) 0.2 (S4)
11	S3 deliver, collect	Line flush, pressurize, vent	10	0.02
12	S3 extract	—	20	—
13	S3 collect	Line flush, pressurize, vent	12	—
14	S3 deliver, collect	Line flush, pressurize, vent	18	0.06
15	S3 extract	Line flush, pressurize, vent	15	—
16	S3 collect	Line flush, pressurize, vent	12	—
17	S3 deliver, collect	Line flush, pressurize, vent	18	0.06
18	S3 extract	Line flush, pressurize, vent	15	—
19	S3 collect	Line flush, pressurize, vent	12	—
20	S3 deliver, collect	Line flush, pressurize, vent	18	0.06
21	S3 extract	Line flush, pressurize, vent	15	—
22	S3 collect	Line flush, pressurize, vent	14	—
23	Delay	Line flush, pressurize, vent	2	—
24	Vacuum	Line flush, pressurize, vent	40	—
25	Pressurize	Line flush, pressurize, vent	2	—
26	Vacuum	Line flush, pressurize, vent	50	—
27	Pressurize	Vacuum	2	—
28	Pressurize, vent; R3 pressurize, vent	Vacuum	6	—
29	R3 deliver	Vacuum	40	5 cm³/min (gas)
30	R3 deliver	Pressurize	2	—
31	R3 deliver	Pressurize, vent; R4 pressurize, vent	6	—
32	R3 deliver	R4 deliver	8	0.2
33	R3 deliver	Line flush, pressurize, vent	3	—
34	R3 deliver	Conversion	400	—
35	Pressurize, vent	Conversion	20	—
36	Vacuum	Conversion	40	—
37	Pressurize	Conversion	2	—
38	Vacuum	Conversion	50	—
39	Delay	Conversion	2	—

(*continued*)

TABLE I (*continued*)

Step	Cartridge functions	Flask functions	Step time (sec)	Volume (ml)
40	Pressurize	Conversion	2	—
41	Pressurize, vent; S3 pressurize, vent	Conversion	6	—
42	S3 deliver	Conversion	18	0.06
43	S3 extract	Conversion	10	—
44	S3 deliver	Conversion	30	0.10
45	Pressurize, vent	Conversion	12	—
46	Vacuum	Conversion	40	—
47	Pressurize	Conversion	2	—
48	Vacuum	Conversion	40	—
49	Pressurize	Conversion	2	—
50	Delay	Conversion	2	—
51	Vacuum	Conversion	60	—
52	Pressurize	Conversion	2	—
53	Pressurize, vent; R2 pressurize, vent	Conversion	6	—
54	R2 deliver	Conversion	100	5 cm³/min (gas)
55	Pressurize, vent; R1 pressurize, vent	Conversion	6	—
56	R1 deliver	Conversion	4	0.03
57	Pressurize, vent	Conversion	40	—
58	R2 deliver	Conversion	400	5 cm³/min (gas)
59	Pressurize, vent	Conversion	20	—
60	Vacuum	Conversion	40	—
61	Pressurize	Conversion	2	—
62	Vacuum	Conversion	40	—
63	Pressurize	Conversion	2	—
64	Pressurize, vent; R1 pressurize, vent	Conversion	6	—
65	R1 deliver	Conversion	4	0.03
66	Pressurize, vent	Conversion	40	—
67	R2 deliver	Conversion	500	5 cm³/min (gas)
68	Pressurize, vent	Conversion	20	—
69	Vacuum	Line flush, pressurize, vent	40	—
70	Pressurize	Line flush, pressurize, vent	2	—
71	Vacuum	Line flush, pressurize, vent	40	—
72	Pressurize	Line flush, pressurize, vent	2	—
73	Vacuum	Line flush, pressurize, vent	60	—
74	Pressurize	Line flush, pressurize, vent	2	—
75	Pressurize, vent; S1 pressurize, vent	Line flush, pressurize, vent	6	—
76	S1 deliver	Line flush, pressurize, vent	14	0.06
77	S1 extract	Line flush, pressurize, vent	20	—
78	S1 deliver	Line flush, pressurize, vent	60	0.3

TABLE I (*continued*)

Step	Cartridge functions	Flask functions	Step time (sec)	Volume (ml)
79	Pressurize, vent; S2 pressurize, vent	Vacuum	6	—
80	S2 deliver	Vacuum	12	0.06
81	S2 extract	Vacuum	20	—
82	S2 deliver	Pressurize	2	1.2 ml total (steps 82–94)
83	S2 deliver	Vacuum	40	—
84	S2 deliver	Pressurize	2	—
85	S2 deliver	Pressurize, vent; S4 pressurize, vent	6	—
86	S2 deliver	S4 deliver	8	0.2
87	S2 deliver	Line flush, pressurize, vent	10	—
88	S2 deliver	Line flush, collect	24	—
89	S2 deliver	S4 deliver	2	0.05
90	S2 deliver	Line flush, pressurize, vent	8	—
91	S2 deliver	Line flush, collect	20	—
92	S2 deliver	S4 deliver	40	1.0
93	S2 deliver	Line flush, waste	20	—
94	S2 deliver	Vacuum	60	—
95	Pressurize, vent	—	12	—
96	Vacuum	—	40	—
97	Pressurize	Collector step	2	—
98	Vacuum	—	40	—
99	Pressurize	—	2	—
100	Vacuum	—	60	—

vacuum application to both chambers. The vacuum stages are generally split into segments interspersed by short pressurization steps. This results in faster removal of volatile material than can be achieved with a single, long vacuum step. Both the reaction chambers and the reagent/solvent reservoirs are pressurized and vented prior to reagent/solvent delivery. This provides accurate, reproducible delivery rates and prevents reverse flow of gases in the instrument. Reagent/solvent deliveries are followed by argon purges of the delivery lines to remove the bulk of the chemicals prior to subsequent steps such as vacuum application. The reaction cartridge and flask are thermostatted at 44° and 50°, respectively.

Coupling. The coupling stage is initiated by addition of a small aliquot of reagent 1 that is sufficient to soak the sample disk thoroughly. Excess reagent and heptane are blown off the disk by a brief argon purge. Then,

reagent 2 vapor is blown through the cartridge to achieve and maintain an alkaline pH in the sample film. After a brief vacuum stage, the reagent 1 and 2 deliveries are repeated.

Postcoupling Solvent Extractions. After the second reagent 2 delivery is completed, the cartridge is evacuated to remove most of the remaining phenylisothiocyanate and coupling base. The remaining reagents and by-products are removed by extraction first with benzene and then with ethyl acetate. The solvent extractions begin with addition of just enough solvent to fill the cartridge chamber. Then, the solvent delivery is interrupted to allow time for the liquid to penetrate the sample film before the major portion of the solvent is delivered.

Cleavage. Cleavage, effected by delivery of reagent 3 vapor through the cartridge, is done in two stages. The first of the two stages is considerably briefer than the second. It lasts long enough to allow cleavage of most amino acid residues to approach completion (>90%) but is brief enough to minimize destruction of the more unstable anilinothiazolinones. The second cleavage generally lasts long enough to allow completion (>99%) of the cleavage of all residues except certain prolyl residues followed by amino acids with bulky side chains. Complete cleavage of these residues, if their presence at certain positions in a sequence is known or suspected, can be effected by doubling or tripling the duration of the second cleavage step.[7] The second cleavage stage can be omitted to decrease cycle time if small- or medium-size peptides are being sequenced.

Postcleavage Solvent Extractions. After each of the cleavage stages is completed, the cartridge is evacuated to remove cleavage acid vapor. Then, the anilinothiazolinones are removed by extraction with 1-chlorobutane. The extractions begin with addition of just enough solvent to fill the cartridge chamber. Then, the solvent delivery is interrupted to allow time for the liquid to penetrate the sample film before the major portion of the solvent is delivered. Solvent extraction after the first cleavage stage is directed to the conversion flask; solvent extraction after the second cleavage stage is directed to the waste bottle.

Postcleavage Base Treatment. After completion of the second cleavage stage and its solvent extraction, the protein can be treated with reagent 2 vapor to reverse N → O acyl shifts at seryl and threonyl residues that contribute to splitting of the peptide chain and creation of background signals.[8] This procedure can be omitted to decrease cycle time if small- to medium-sized peptides are being sequenced.

[7] W. E. Brandt, P. Edman, A. Henschen, and C. von Holt, *Hoppe-Seyler's J. Physiol. Chem.* 357, 1505 (1976).

[8] J. Thomsen, D. Bucher, K. Brunfeldt, E. Nexo, and H. Olesen, *Eur. J. Biochem.* 69, 87 (1976).

>PhNCS Conversion. The 1-chlorobutane extract following the first cleavage stage is directed into the conversion flask. A small volume of methanol is added to the conversion flask before the 1-chlorobutane extract in order to remove trifluoroacetyl groups from serine and threonine hydroxyl groups and thereby maximize recovery of >PhNCS serine and threonine.[9] In order to confine the sample to the lower tip of the miniaturized flask, the anilinothiazolinones are extracted from the cartridge disk with several small volumes of 1-chlorobutane delivered sequentially with brief periods of stopped flow. Continuous drying of the flask contents during the solvent delivery and delay steps is effected by flushing the flask with argon in order to minimize the time between extraction from the disk and addition of reagent 4 to the flask. The last traces of solvent are removed by a brief evacuation of the flask before the reagent 4 delivery.

The conversion reaction requires 25–30 min at 50° to proceed to completion. After this time, the bulk of the reagent is removed by flushing the flask with argon, and the last traces are removed by evacuation. The >PhNCS amino acids are transferred from the flask to the fraction collector by 200- and 50-μl aliquots of methanol. Then, the flask is washed with 1 ml of methanol (dumped to the waste bottle) to remove any >PhNCS residues that splash onto the upper wall of the flask.

Sample Loading. Polybrene (Aldrich Chemical Company, Milwaukee, Wisconsin), used as a carrier to retain protein or peptide on the disk, must be precycled through the sequenator program before it is suitable for microsequencing applications.[10] This precycling removes soluble impurities from the Polybrene and also blocks sites on the polymer that otherwise could react with amino-terminal groups of the protein sample. The cartridge is disassembled as described above, and a new glass fiber disk is placed in its recess in the upper glass cylinder. An aqueous solution (30 μl) of Polybrene (60 mg/ml) and glycylglycine (1 μmol/ml) is spotted onto the disk and dried under vacuum. After reassembly of the cartridge, the Polybrene is subjected to 4–8 cycles of a modified sequencing program in which there is (*a*) a single cleavage stage with the subsequent solvent extraction directed to the waste bottle, and (*b*) a coupling stage with only one addition of reagents 1 and 2. The precycling must begin at the coupling, rather than cleavage, stage in order to prevent loss of the Polybrene from the disk.

[9] M. J. Horn and A. G. Bonner, *in* "Solid Phase Methods in Protein Sequence Analysis" (A. Previero and M.-A. Coletti-Previero, eds.), p. 163. North-Holland Publ., Amsterdam, 1977.

[10] M. W. Hunkapiller and L. E. Hood, *Biochemistry* 17, 2124 (1978).

After completion of the Polybrene precycling, the cartridge is disassembled, the protein solution (25–30 μl) is spotted onto the disk and dried under vacuum, and the cartridge is reassembled. The sequencing program is started at the second cleavage stage and then carried through two coupling stages without the normally intervening cleavage stages on the first cycle to ensure complete coupling at the protein's amino terminus.

>PhNCS Amino Acid Analysis. The >PhNCS amino acids produced in the gas phase sequenator can be analyzed by any of the methods commonly used in protein sequencing. However, if the amount of protein being sequenced is less than a few nanomoles, high-performance liquid chromatography is the method of choice. The protocol that has been used with the gas-phase sequenator in our laboratory is described in detail in this volume [43]. It is suitable for amounts of protein as little as 5 pmol.

TABLE II
SAMPLE OF PROTEINS SEQUENCED ON GAS PHASE SEQUENATOR

Protein	Molecular weight	Residues identified	Amount	
			pmol	μg
Angiotensin II	1,000	8	500	0.5
Angiotensin II	1,000	6	50	0.05
Somatostatin	1,600	14	1,200	2.0
Insulin, B chain	3,400	30	260	1.0
Aplysia neuropeptide B	3,800	31	500	2.0
Dynorphin	2,000	14	20	0.04
Dynorphin precursor	3,200	31	200	0.6
Myoglobin	17,500	90	10,000	175
Myoglobin	17,500	22	5	0.09
Drosophila larval cuticle protein[a,b]	18,000	55	850	15
Aplysia membrane phosphoprotein[a,b]	22,000	23	15	0.3
Human histocompatibility antigen				
HLA-DR, α chain[b]	32,000	49	700	23
HLA-DR, β chain[b]	26,000	39	500	13
Mouse immune response antigen E_β^k[a–c]	25,000	21	20	0.5
Human erythropoietin[d]	44,000	40	100	4
Human melanoma cell surface antigen[a,b]	95,000	13	60	5.5
Eel acetylcholine receptor α subunit[a,b]	40,000	68	400	16
Calf acetylcholine receptor α subunit[a,b]	42,000	35	50	2

[a] Electrophoretically eluted from Coomassie Blue-stained, SDS–polyacrylamide gels.
[b] Integral membrane protein.
[c] Purified by isoelectric focusing in polyacrylamide gels containing urea.
[d] Sixty percent carbohydrate by weight.

cling, the cartridge is disas-
otted onto the disk and dried
ed. The sequencing program
n carried through two coupl-
cleavage stages on the first
tein's amino terminus.
CS amino acids produced in
y any of the methods com-
r, if the amount of protein
es, high-performance liquid
protocol that has been used
ory is described in detail in
f protein as little as 5 pmol.

PHASE SEQUENATOR

Residues identified	Amount pmol	Amount μg
8	500	0.5
6	50	0.05
14	1,200	2.0
30	260	1.0
31	500	2.0
14	20	0.04
31	200	0.6
90	10,000	175
22	5	0.09
55	850	15
23	15	0.3
49	700	23
39	500	13
21	20	0.5
40	100	4
13	60	5.5
68	400	16
35	50	2

ined, SDS-polyacrylamide gels.

:ls containing urea.

Results

Performance of the gas-phase sequenator has been evaluated with a variety of proteins and peptides of known and unknown sequence. Some of the results of these sequencing experiments (many from Hewick *et al.*[4]) are shown in Table II. Proteins can be analyzed using as little as 5 pmol of sample; peptides can be sequenced with as little as 50 pmol of sample. Both hydrophobic proteins and peptides, including integral membrane proteins, can be sequenced. Proteins purified by SDS-polyacrylamide gel electrophoresis (PAGE) and by electrofocusing in polyacrylamide gels can be analyzed after the proteins are removed from the gels by electrophoretic elution (this volume [17]).

The efficiency of the Edman degradation on the gas-phase sequenator, as measured by the repetitive cycle yield, is exceptionally high. Repetitive yields for sequenator runs with 10 nmol of sperm whale apomyoglobin are around 98% and with as little as 10 pmol are still as high as 92%.[4] This has permitted sequenator runs of 70–90 residues using 10 nmol of protein and 15–25 residues using 10 pmol of protein. The latter amounts can easily be obtained from two-dimensional PAGE; while the amount of sequence information obtained directly is limited, it is often sufficient to provide identification of the protein or the sequence of cDNA probes for use in gene cloning experiments.

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[37] Radiochemical Sequence Analysis of Biosynthetically Labeled Proteins

By JOHN E. COLIGAN, FREDERICK T. GATES III, EDWARD S. KIMBALL, and W. LEE MALOY

Background

Many protein molecules, particularly those that are membrane-bound, are not readily available in the amounts necessary for amino acid sequence determination by traditional methodology. Consequently, effort

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lamellar bodies disperse, and prothylakoids eventually develop into THYLAKOIDS.

proliferative kidney disease (PKD) A FISH DISEASE which can be economically important in young salmonid fish. Symptoms include exophthalmia, anaemia, abdominal swelling and kidney hypertrophy; mortality rate: 10–95%. PKD is caused by an unclassified protozoon which apparently has affinities with members of the MYXOZOA [JP (1985) *32* 254–260].

Proliferobasidium See BRACHYBASIDIALES.

L-proline biosynthesis See Appendix IV(a).

proloculus See FORAMINIFERIDA.

promastigote A form assumed by the cells of many species of the TRYPANOSOMATIDAE (q.v.) during at least certain stages of their life cycles.

prometaphase See MITOSIS.

Promicromonospora A genus of asporogenous bacteria (order ACTINOMYCETALES, wall type VI) which occur e.g. in soil. The organisms form a yellow fragmenting mycelium. GC%: ca. 73. Type species: *P. citrea*. [Book ref. 73, pp. 53–54.]

promiscuous plasmids Those CONJUGATIVE PLASMIDS (sense 1) — e.g. IncP1 plasmids — which are capable of self-transmission between bacteria of a wide range of different species and genera.

promitochondrion See MITOCHONDRION.

promoter In a DNA strand: a nucleotide sequence which is recognized (directly or indirectly) and bound by a DNA-dependent RNA POLYMERASE (RPase) during the initiation of TRANSCRIPTION. Transcription is initiated at a position (the *start point* or *start site*) which is usually within the promoter sequence; the first nucleotide to be transcribed is designated +1, nucleotides *downstream* of this position (i.e., those in the direction of RNA elongation: 5′-to-3′ with respect to the RNA) are numbered +2, +3, +4 etc. and nucleotides in the opposite (*upstream*) direction are numbered −1, −2, −3 etc.

In eubacteria there are several RPase holoenzymes (differing in their SIGMA FACTORS), each of which recognizes a distinct class of promoter. In *Escherichia coli* most transcription is carried out by $E\sigma^{70}$; the enzyme binds to a region of DNA extending from ca. 50 bp upstream to ca. 20 bp downstream of the start point. Comparison of the sequences of many promoters recognized by $E\sigma^{70}$ has revealed that certain short sequences are more or less conserved: a CONSENSUS SEQUENCE, TATAAT (the *Pribnow box* or *−10 sequence*), is centred ca. 10 bp upstream from the start point, and another consensus sequence, TTGACA (the *−35 sequence*) is centred ca. 35 bp upstream of the start point; promoters which lack one or the other of these sequences apparently cannot be recognized by $E\sigma^{70}$ without the participation of other proteins (see e.g. CATABOLITE REPRESSION). The start point itself is usually a purine, often an adenine residue in the sequence CAT.

Promoters recognized by $E\sigma^{70}$ in *E. coli* differ to varying extents from the theoretical 'consensus promoter', and may also differ from one another in functional efficiency (efficient promoters being described as 'strong', inefficient ones as 'weak'). Efficiency may be affected e.g. by changes in the consensus sequences or in the transcribed region downstream of the start point. Changes at one site may be compensated for by changes at another: thus, promoters with the same efficiencies may have different base sequences; furthermore a (synthetic) promoter containing the theoretical consensus sequences has been found to be relatively inefficient. [EMBO (1986) *11* 2987–2994, 2995–3000.]

Promoters of other classes (recognized by different RPase holoenzymes) in bacteria also typically have two short, distinctive conserved sequences upstream from the start point; however, the actual sequences vary from one class of promoter to another. For example, in *Bacillus subtilis* the main holoenzyme, $E\sigma^{43}$, recognizes the same type of promoter as does the $E\sigma^{70}$ of *E. coli*, but $E\sigma^{37}$ (see SIGMA FACTOR) recognizes a consensus sequence AGGATTTNA (N = any nucleotide) in the −35 region and GGAATTNTTT in the −10 region; the corresponding sequences for $E\sigma^{29}$ are TTNAAA and CATATT. (The phage T4 $E\sigma^{gp55}$ recognizes a promoter which apparently lacks a −35 sequence; in promoters recognized by the $E\sigma^{gpuvA}$ of e.g. *Klebsiella* a −35 sequence is apparently absent, but there is a consensus sequence in the −20 region.)

In eukaryotes, promoters are not recognized directly by the RNA polymerases; transcription initiation factors (TIFs) first bind to a promoter to form a *preinitiation complex*, and only then does an RPase bind to form an *initiation complex*. Promoters for RPase II generally contain a short consensus sequence, TATA(A/T)A(A/T) (the *TATA box*, *Goldberg–Hogness box*, or *Hogness box*), ca. 25–30 bp upstream from the transcription start point; this appears to be the only well-conserved sequence in RPase II promoters [NAR (1986) *14* 10009–10026], although other sequences upstream of the TATA box may be important sites for the action of promoter-specific TIFs [review: Nature (1985) *316* 774–778]. (See also ENHANCER.) Promoters for RPase III appear to occur *within* the associated gene, i.e., *downstream* of the start point; binding of the RPase at the promoter leads

to initiation of transcription at a particular distance upstream of the binding site.

promoter control See OPERON.

promycelium See METABASIDIUM.

Pronase (proprietary name) A mixture of endopeptidases and exopeptidases (including carboxypeptidase(s) and aminopeptidases) which has non-specific protease activity. It is obtained from *Streptomyces griseus*.

Prontosil An early sulphonamide, first used clinically in the 1930s; its activity was due to its breakdown in vivo to sulphanilamide. It has been superceded by the more effective, less toxic modern SULPHONAMIDES.

pronucleus (1) The haploid nucleus of a gamete. (2) (*protozool.*) See AUTOGAMY and CONJUGATION.

proof-reading (*mol. biol.*) A system in which the accuracy of a process is increased by the removal of 'errors' immediately after they have occurred: see e.g. DNA REPLICATION and PROTEIN SYNTHESIS.

propagative viruses See CIRCULATIVE TRANSMISSION.

propagule Any disseminative unit of an organism, e.g. a spore, a mycelial fragment.

propamidine An aromatic DIAMIDINE used as an antiseptic; it is active mainly against asporogenous Gram-positive bacteria and certain fungi.

propeller loop fermenter A LOOP FERMENTER in which a motor-driven propeller promotes circulation in the column by impelling the culture vertically up (or down) the DRAFT TUBE.

proper margin (excipulum proprium) (*lichenol.*) *Non*-lichenized (i.e., wholly fungal) tissue forming the excipular margin (rim) of a lichen APOTHECIUM. A proper margin is often the same colour as the hymenial disc. (cf. THALLINE MARGIN; see also LECIDEINE APOTHECIUM.)

properdin A γ-globulin (MWt ca. 220,000) which occurs in normal serum (ca. 25 μg/ml). Properdin promotes the *alternative pathway* of COMPLEMENT FIXATION by stabilizing the C3 convertase (C3bBb) in the presence of specific activators of this pathway.

prophage See LYSOGENY.

prophage immunity See SUPERINFECTION IMMUNITY.

prophase See MITOSIS and MEIOSIS.

prophylaxis Measure(s) taken to prevent the occurrence of disease — e.g. DISINFECTION, IMMUNIZATION. (See also PROTECTANT.)

propiconazole (1-[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2,2-yl-methyl]-1H-1,2,4-triazole; trade names: e.g. Radar, Tilt) An agricultural AZOLE ANTIFUNGAL AGENT which has both contact and systemic action against a wide range of plant pathogenic fungi; it has both eradicant and protectant properties against e.g. cereal diseases such as eyespot, net blotch, powdery mildew, rhynchosporium, septoria, yellow and brown rusts, etc. It may be mixed with e.g. CARBENDAZIM to give a preparation with an even broader antifungal spectrum.

propidium A phenanthridine trypanocidal agent and INTERCALATING AGENT (apparent unwinding angle: 26°).

β-propiolactone (BPL) A water-miscible, non-inflammable cyclic ether (b.p. 155°C) used e.g. (in vapour form) as a surface STERILANT; it has low powers of penetration, needs a high relative humidity (ca. 70%) for maximum activity, and may be poorly effective against e.g. dried spores (cf. ETHYLENE OXIDE). BPL acts as an ALKYLATING AGENT, substituting proteins etc with propionic acid residues. Above 25°C it is readily hydrolysed to β-hydroxypropionic acid (hydracrylic acid); under acid conditions it forms an open-chain ester-linked polymer. (N.B. BPL is irritant and may be carcinogenic.)

Propionibacterium A genus of Gram-positive, asporogenous, chemoorganotrophic, anaerobic bacteria which occur e.g. in dairy products (see e.g. CHEESE-MAKING) and on the human skin, and which form propionic acid as a main product in the PROPIONIC ACID FERMENTATION of hexoses or lactate. Cells: non-motile, pleomorphic, branched or unbranched rods or coccoid forms which may exhibit a 'Chinese letter' arrangement in stained preparations; some form pigments. The organisms can be cultured e.g. on yeast extract–lactate–peptone media. GC%: ca. 57–67. Type species: *P. freudenreichii*.

P. acnes (formerly *Corynebacterium parvum*). The organisms ferment glucose (but not maltose or sucrose), do not hydrolyse aesculin, and are indole-positive; they digest casein and liquefy gelatin. [Extracellular proteolysis: JAB (1983) *54* 263–271.] Colonies older than ca. 4 days may become reddish. (See also ACNE and SKIN MICROFLORA.)

Other species: *P. acidi-propionici*; *P. freudenreichii* (which incorporates *P. shermanii*) (see also PYROPHOSPHATE); *P. jensenii* (which incorporates *P. peterssonii* and *P. raffinosaceum*); *P. thoenii*. [Book ref. 46, pp. 1894–1902.]

propionic acid fermentation A FERMENTATION (sense 1), carried out e.g. by *Propionibacterium* spp, *Clostridium propionicum* and *Megasphaera elsdenii*, in which e.g. glucose and/or lactate yield propionic acid and acetic acid as the main end products. *Propionibacterium* spp ferment glucose or lactate via succinate [Appendix III(h)]; when lactate is the substrate the reduction of (endogenous) fumarate generates proton motive force (pmf), permitting ATP synthesis by electron transport

pH.) (b) SpA-containing staphylococci can bind to specific (IgG) antibodies (via their Fc portions) and will then agglutinate on exposure to the homologous antigen (CO-AGGLUTINATION); such antibody-coated staphylococci can be used e.g. in slide co-agglutination tests for detecting and/or identifying bacteria, toxins, etc. (c) SpA may be bound to red blood cells (e.g. with glutaraldehyde) and used e.g. to detect IgG on the surface of lymphocytes (by rosette formation). (d) SpA conjugated with an enzyme (e.g. β-lactamase, horseradish peroxidase) can be used e.g. for the assay of IgG in serum. Antigen is immobilized, incubated with the serum, and any homologous antibody binding to the antigen is detected with the SpA–enzyme conjugate; the amount of SpA bound, and hence the amount of homologous IgG in the serum, is determined by an assay of the enzymic activity (e.g. PADAC hydrolysis for β-lactamase conjugates) [PTRSLB (1983) *300* 399–410].

[Properties and applications of protein A: Book ref. 44, pp. 429–480.]

protein G A cell wall protein from group G streptococci; it resembles staphylococcal PROTEIN A in binding specifically to the Fc region of an IgG molecule, but it binds a broader range of IgG subclasses. [Structure of the IgG-binding regions of protein G: EMBO (1986) *5* 1567–1575.]

protein i See PRIMOSOME.

protein kinase An enzyme which phosphorylates particular amino acid residues in a protein. The functions of certain proteins are regulated by phosphorylation/dephosphorylation both in eukaryotic cells (see e.g. ONCOGENE) and in *Escherichia coli* (see e.g. TCA CYCLE (isocitrate dehydrogenase)). (See also CYCLIC AMP.)

protein n (also n′, n″) See PRIMOSOME.

protein synthesis The biosynthesis of a protein is a complex process in which a nucleotide sequence in a DNA (or, in some viruses, RNA) molecule determines the sequence of amino acids in the protein: see GENETIC CODE. In cells, the first step is the synthesis (TRANSCRIPTION) of a messenger RNA (mRNA) molecule on the DNA template; the (mature) mRNA molecule (see MRNA) then functions as a template for the assembly of the polypeptide chain (a process known as *translation*). However, amino acids cannot recognize their specific codons in the mRNA directly, and each must be linked to a specific transfer RNA (tRNA, q.v.) which contains the correct anticodon (see GENETIC CODE). The 'charging' of a tRNA with its specific amino acid is achieved in a two-step reaction: *activation*, in which the amino acid reacts with ATP in the presence of a specific *aminoacyl-tRNA synthetase* to form an enzyme-bound aminoacyl-AMP complex (PPi being released), and *transfer*, in which the aminoacyl group is transferred to the 2′ or 3′ position of the terminal adenosine residue in the appropriate tRNA (AMP and the synthetase being released). (See also MUPIROCIN.) In eukaryotes, but not in bacteria, aminoacyl-tRNA synthetases occur in high-MWt multienzyme complexes [review: Bioch. J. (1986) *239* 249–255].

Interaction between charged tRNAs (aa-tRNAs) and the corresponding codons in the mRNA, as well as subsequent peptide bond formation, are mediated by the RIBOSOMES. Each ribosome has two binding sites for charged tRNA: the *A site* (= acceptor or entry site) and the *P site* (= peptidyl or donor site). Essentially, a ribosome binds to the mRNA and moves along it such that each codon in turn is exposed to the A site; all incoming aa-tRNAs (*except* the first) enter the A site.

The process of translation is divided into three main phases: initiation, elongation, and termination.

Initiation. In all known systems, the initiator codon (usually AUG, but occasionally e.g. GUG, UUG or AUU) is recognized by a distinct *initiator tRNA* ($tRNA_i$) which is charged with methionine (Met). In e.g. *Escherichia coli* there are two tRNAs specific for Met: $tRNA_m^{Met}$ and $tRNA_f^{Met}$; only the latter can function as a $tRNA_i$. The charged $tRNA_f^{Met}$, Met-$tRNA_f$, is usually formylated at the α-amino group of the methionyl residue to give *N*-formylmethionyl-$tRNA_f$ (fMet-$tRNA_f$); this reaction requires N^{10}-formyltetrahydrofolate. Met-$tRNA_m$ cannot be formylated and is involved in the incorporation of Met during polypeptide chain elongation. Thus, in *E. coli* (and most other prokaryotes) *N*-formylmethionine is usually the first (N-terminal) amino acid to be incorporated in the polypeptide chain. A similar system operates in mitochondria and chloroplasts, but in eukaryotic cytoplasm — and apparently in at least some archaebacteria (e.g. *Halobacterium cutirubrum*) — the Met-$tRNA_i$ is not formylated (thus, the N-terminal amino acid is methionine).

In *E. coli* a specific protein *initiation factor*, IF-2, is necessary for translation initiation. According to one model, IF-2 and fMet-$tRNA_i$ interact in the presence of GTP to form a *preinitiation complex* which then binds to the 30S ribosomal subunit, the tRNA occupying the (incomplete) ribosomal P site. (The preinitiation complex could bind to the 30S subunit before or after the 30S subunit binds to the mRNA.) However, according to an

alternative model, IF-2 may act directly on the 30S subunit to stimulate template-dependent 30S–aa-tRNA binding [see e.g. FEBS (1986) *207* 198–204]. Two other protein initiation factors, IF-3 and IF-1, also bind to the initiation complex. IF-3 is necessary for the binding of the 30S subunit to the mRNA (as well as for the dissociation of 70S ribosomes into their 30S and 50S subunits and, hence, the provision of 30S subunits). IF-1 apparently stimulates the activity of IF-2.

In bacteria, the ribosome-binding site on an mRNA molecule includes a sequence (ca. 3–12 nt upstream of the AUG codon) of 3–9 purine nucleotides, the *Shine–Dalgarno sequence*, which is complementary to a sequence near the 3′ end of the 16S rRNA in the 30S subunit; this sequence apparently serves to align the ribosomal P site with the correct initiation codon. IF-1 is released, and the 50S ribosomal subunit then binds to the 30S-initiation complex (with concomitant release of IF-3) to form the complete 70S ribosome ('monosome'). Finally, GTP is hydrolysed (in an IF-2-dependent reaction) to GDP and Pi, IF-2 being released.

Initiation can apparently also occur with a 70S monosome; this may happen e.g. when initiation occurs at an internal initiator codon in a polycistronic mRNA, the ribosome terminating synthesis of one polypeptide and initiating synthesis of the next without dissociating from the mRNA.

In eukaryotic cytoplasm, initiation usually occurs at the AUG nearest the 5′ end of the mRNA, and generally requires a capped mRNA (see MRNA) for maximum efficiency; there is apparently no sequence equivalent to the Shine–Dalgarno sequence, and at least 9 initiation factors are required.

In addition to their function as initiator codons, AUG, GUG and UUG code for methionine, valine and leucine, respectively; it is not clear how an initiator codon is distinguished from internal (or out-of-phase) codons. Although in bacteria the Shine–Dalgarno sequence is believed to play a role in this process, it is apparently not by itself sufficient to determine the initiation site; some genes in *E. coli* apparently lack a Shine–Dalgarno sequence. Various models have been proposed to account for the specific recognition of an initiator codon. For example, it has been suggested that the translation initiation region in mRNA contains a 'mosaic' of sequences complementary to one or more regions in the D and T loops, as well as in the A loop, of the $tRNA_i$ (and in the 16S rRNA), and furthermore that unusual initiator codons such as GUG and UUG may be recognized by sequences in the T loop rather than by a conventional anticodon in the A loop of the $tRNA_i$ [PNAS (1985) *82* 4587–4591].

Once initiation is complete and polypeptide chain elongation is under way, the formyl group at the N-terminal end may be removed by a *deformylase*; the methionine residue, and sometimes other residues, may also be removed. (See also SIGNAL HYPOTHESIS.)

Antibiotics which interfere with translation initiation include e.g. AURINTRICARBOXYLIC ACID, kasugamycin (see AMINOGLYCOSIDE ANTIBIOTICS), and PACTAMYCIN.

Elongation of the polypeptide chain is believed to occur in three successive steps repeated at each codon. In the first (*decoding*) step the aa-tRNA which corresponds to the second codon (adjacent to the initiator codon) enters the vacant ribosomal A site. In *E. coli* this step requires the protein *elongation factor T* (EF-T; 'transfer factor') which has two components: EF-Tu and EF-Ts. (EF-Tu is an abundant protein in *E. coli*; it is encoded by two nearly identical, unlinked genes, *tufA* and *tufB*, the two gene products (EF-TuA and EF-TuB) differing only in the C-terminal amino acid: glycine for EF-TuA, serine for EF-TuB.) EF-Tu–EF-Ts interacts with GTP, EF-Ts being displaced and EF-Tu–GTP being formed. EF-Tu–GTP can complex with any aa-tRNA (*except* fMet-$tRNA_f$) to form the ternary complex aa-tRNA–EF-Tu–GTP; this complex then binds at the A site of a ribosome in which the P site is occupied by an fMet-$tRNA_f$ (or, later in elongation, by a peptidyl-tRNA). GTP is hydrolysed and EF-Tu–GDP and Pi are released. EF-Ts displaces the GDP to reform EF-Tu–EF-Ts which can then react with GTP to repeat the cycle.

In the second step, *transpeptidation*, a peptide bond is formed between the α-amino group of the aa-tRNA in the A site and the carboxyl group of the fMet-$tRNA_f$ in the P site; this leaves uncharged $tRNA_f^{Met}$ in the P site and the dipeptidyl-tRNA (fMet–aa-$tRNA^{aa}$) in the A site. Transpeptidation, which does not require elongation factors, is catalysed by a *peptidyl transferase* activity in the 50S ribosomal subunit.

In the third step, *translocation*, the ribosome moves relative to the mRNA–dipeptidyl-tRNA by three nucleotides (i.e., one codon) in the 5′-to-3′ direction; concomitantly, the uncharged $tRNA_f^{Met}$ may be ejected, the dipeptidyl-tRNA enters the P site, and the vacated A site is brought into juxtaposition with the next codon. This is the 'two-site' model for translocation. However, evidence has been obtained for a 'three-site' model for translocation in *E. coli* and e.g. '*Halobacterium halobium*' [MGG (1986) *204* 221–228, q.v. for refs]. According to this

model, tRNAs remain bound to the ribosome both before and after translocation, i.e., deacylated tRNA is not released from the P site during translocation; instead, it is transferred to a third ribosomal site, designated the *E site*, concomitantly with translocation. Release of the deacylated tRNA from the E site is triggered by the entry into the A site of the next aa-tRNA.

Translocation in *E. coli* requires *elongation factor G* (EF-G) and is accompanied by GTP hydrolysis — probably catalysed by EF-G in the presence of the ribosome; GTP hydrolysis is apparently not required for translocation, but may be necessary to effect detachment of EF-G from the ribosome [JMB (1986) *189* 653–662]. In eukaryotes, the factor equivalent to the EF-G of *E. coli* is eEF-1; eEF-1 differs from EF-G in being susceptible to ADP-ribosylation by DIPHTHERIA TOXIN (q.v.). Archaebacteria also characteristically have an EF which is susceptible to diphtheria toxin [Book ref. 157, pp. 379–410].

The three elongation steps are repeated cyclically at each codon, the growing peptide chain always being transferred to the α-amino group of the incoming aa-tRNA in the A site. As each ribosome passes along the mRNA, another ribosome can bind to the vacated initiation site and initiate the translation of another polypeptide chain. Thus, a given mRNA generally carries many ribosomes situated at intervals along its length, forming a structure known as a *polyribosome* (*polysome*). (A single ribosome is sometimes called a *monosome*.)

Many antibiotics act by interfering with particular stages in the elongation process in particular organisms: see e.g. AMINOGLYCOSIDE ANTIBIOTICS; ANISOMYCIN; CHLORAMPHENICOL; CYCLOHEXIMIDE; FUSIDIC ACID; LINCOSAMIDES; MACROLIDE ANTIBIOTICS; POLYENE ANTIBIOTICS (b); PUROMYCIN; SPARSOMYCIN; STREPTOGRAMINS; TETRACYCLINES; TIAMULIN; THIOSTREPTON; VIOMYCIN.

Termination occurs when the ribosome reaches a specific termination codon (UAA, UAG or UGA in *E. coli*: see GENETIC CODE). These codons are recognized not by tRNAs but by protein *release factors* (RFs): RF-1 recognizes UAA and UAG, RF-2 recognizes UAA and UGA. Binding of an RF to a termination codon results in the hydrolysis of the ester bond between the tRNA and the polypeptide chain (catalysed by the ribosomal peptidyl transferase) and hence the release of the completed polypeptide. A third factor, RF-3 (= 'S protein'), apparently stimulates the activities of RF-1 and RF-2.

In eukaryotic cytoplasm there is apparently only one RF (eRF) which can recognize any of the three termination codons: eRF requires GTP for activity.

Proof-reading can occur at various stages during protein synthesis in bacteria, serving to maintain a high level of translational accuracy. Proof-reading can occur e.g. at the level of amino acid selection by aa-tRNA synthetases. Synthetase specificity is not always high enough to prevent binding of the 'wrong' amino acid; a 'wrong' amino acid may be recognized and removed either at the aminoacyl-AMP stage (*pretransfer proof-reading*) or at the aa-tRNA stage (*post-transfer proof-reading*) [see e.g. NAR (1986) *14* 7529–7539]. Proof-reading can also occur during the elongation phase of protein synthesis; aminoglycosides such as STREPTOMYCIN are believed to exert their effect on translational accuracy by interfering with this proof-reading system.

proteinase (1) *Syn.* protease (see PROTEASES). (2) *Syn.* endopeptidase (see PROTEASES).

proteinase K A non-specific PROTEASE obtained from *Tritirachium album*. (cf. PRONASE.)

proteose A soluble product of protein hydrolysis; proteoses are not coagulated by heat but are precipitated in saturated $(NH_4)_2SO_4$.

proter (*ciliate protozool.*) The anterior of the two cells formed during HOMOTHETOGENIC binary fission. (cf. OPISTHE.)

Proteromonadida An order of parasitic protozoa (class ZOOMASTIGOPHOREA). The cells have one or two pairs of flagella, without paraxial rods, and a single mitochondrion which lacks a kinetoplast; cysts are formed. Genera: e.g. *Karatomorpha*, *Proteromonas*.

Proteromonas See PROTEROMONADIDA.

Proteus A genus of Gram-negative bacteria of the tribe PROTEEAE. Cells: ca. 0.4–0.8 × 1–3 μm. Most strains swarm at 37°C, typically forming characteristic concentric zones of growth on the moist surface of an agar or gelatin medium (see SWARMING and DIENES PHENOMENON). (Some strains may form a spreading, *uniform* film of growth.) Typical reactions: H_2S (in TSI) +ve (delayed in *P. myxofaciens*); gelatin is liquefied at 22°C; lipase +ve; urease +ve; mannose and sugar alcohols are not attacked. Nicotinic acid (but not pantothenic acid) is required for growth. GC%: 38–41. Type species: *P. vulgaris*.

P. inconstans. See PROVIDENCIA.

P. mirabilis. Indole −ve; maltose −ve, ornithine decarboxylase +ve.

P. morganii. See MORGANELLA.

P. myxofaciens. Indole −ve; maltose +ve; ornithine decarboxylase −ve; abundant slime produced e.g. in trypticase–soy broth.

P. rettgeri. See PROVIDENCIA.

P. shigelloides. See PLESIOMONAS.

P. vulgaris. Indole +ve; maltose +ve; ornithine decarboxylase −ve.

abrasion of the cornea by the eyelashes causes ulceration, scarring and impairment/loss of vision. A thin fibrovascular membrane (*pannus*) develops on the surface of the cornea. Lymphoid follicles may develop at the cornea–sclera junction; on healing, these leave characteristic depressions (*Herbert's pits*). The mechanism of pathogenesis is not understood; it has been suggested that it may involve cell-mediated HYPERSENSITIVITY. Chemotherapeutic agents commonly used include e.g. TETRACYCLINES. Laboratory diagnosis typically involves the examination of specimens by e.g. IMMUNOFLUORESCENCE or enzyme immunoassay techniques.

[Book ref. 193, pp. 135–170.]

Trachyspora See UREDINIOMYCETES.

trachytectum *Syn.* EXOSPORIUM (sense 2).

tractellum A (eukaryotic) FLAGELLUM which 'pulls' the cell forwards.

Trager duck spleen necrosis virus See AVIAN RETICULOENDOTHELIOSIS VIRUSES.

***traI* gene** See F PLASMID.

trailer (*mol. biol.*) See MRNA.

***traJ* gene** See F PLASMID.

***traM* gene** See F PLASMID.

trama The sterile (i.e., non-generative) inner tissue of a LAMELLA or DISSEPIMENT or of the 'teeth' of members of the Hydnaceae. (See also BILATERAL TRAMA.)

Trametes A genus of lignicolous fungi of the APHYLLOPHORALES (family Polyporaceae) which form basidiocarps in which the hymenophore is porous. *T. pini* is parasitic on certain trees. For *T. versicolor* see CORIOLUS. (See also XYLANASES.)

***trans*-acting** See CIS-DOMINANCE.

***trans* complementation** See CIS–TRANS TEST.

transaldolase See Appendix I(b) and RMP PATHWAY.

transcapsidation See PHENOTYPIC MIXING.

transcipient A cell which has received DNA from another cell.

transconjugant See CONJUGATION (1b).

transcriptase An RNA POLYMERASE involved in TRANSCRIPTION. (cf. RNA-DEPENDENT RNA POLYMERASE.)

transcription The synthesis of an RNA strand in a process in which ribonucleotide 5'-triphosphates (rNTPs) base-pair sequentially with nucleotides in a template strand and are polymerized in the 5'-to-3' direction (with elimination of PPi) by an RNA POLYMERASE; the template strand is DNA in cells and DNA viruses, RNA in e.g. RNA viruses (see RNA-DEPENDENT RNA POLYMERASE). (cf. REVERSE TRANSCRIPTASE.)

In e.g. *Escherichia coli* (and other eubacteria) *initiation* of transcription begins when the RNA POLYMERASE (RPase) holoenzyme binds to a PROMOTER. The polymerase core enzyme itself has a high affinity for dsDNA and can bind at (apparently) any site to form a stable, 'closed' enzyme–DNA complex in which the DNA strands are not unwound. Interaction of a SIGMA FACTOR with the core enzyme confers on it specificity for a particular class of PROMOTER while greatly reducing its affinity for other DNA sequences. The holoenzyme binds very tightly to its corresponding promoter; initially a 'closed' complex is formed, but this is subsequently converted to an 'open' complex in which a short region of the DNA bound by the enzyme becomes unwound. The first rNTP can then pair with a base on one of the DNA strands (the *start point* or *start site*). A few nucleotides are then incorporated, each being added to the 3'-OH of the preceding nucleotide; the σ factor then dissociates. (The first nucleotide retains its 5'-triphosphate group.)

Elongation is carried out by the RPase core enzyme (possibly in association with the product of the *nusA* gene, a protein which can bind to the core enzyme but not to the holoenzyme); the polymerase moves along the dsDNA, locally unwinding the strands to expose the ssDNA template, 'supervising' the correct base-pairing between incoming rNTPs and the template, and linking the nucleotides in the 5'-to-3' direction (antiparallel to the template) with elimination of pyrophosphate. In this way, a transient RNA–DNA hybrid duplex is formed in the region of the enzyme–DNA complex; as the enzyme proceeds, the RNA peels away from the template and the DNA duplex is restored.

Elongation continues until a specific *termination* signal (*terminator*, *t*) is reached. Terminators in bacteria vary in efficiency and in mechanism of action; secondary structure in the transcript itself appears to be important in effecting termination. In a *rho-independent* ('simple') terminator the RNA transcript of the termination region contains a GC-rich PALINDROMIC SEQUENCE (which can form a stem-and-loop structure) continuous with a run of consecutive uridine (rU) residues at the 3' end. The stem-and-loop structure causes the RPase to pause at the oligo-rU region, and the sequence of rU-dA base pairs (which is relatively unstable) may facilitate the release of the transcript and/or dissociation of the transcription complex. In a *rho-dependent* ('complex') terminator, the transcript may contain a stem-and-loop structure (which is not particularly GC-rich), but there is generally no oligo-rU sequence or any other apparent consensus sequence. In this case termination requires the participation of a protein, the *rho* (ρ) *factor*; the ρ factor is apparently active in hexameric form, has

RNA-dependent NTPase activity (which is necessary for termination), and apparently interacts directly with the RNA transcript. The precise mode of action of ρ is unknown; it has been proposed that there is a ρ recognition site in the RNA, and that termination occurs at a relatively fixed distance downstream of this recognition site [Book ref. 188, pp. 155–178]. The NusA protein also appears to play some role in termination. [Review of transcription termination: Book ref. 60, pp. 123–161.]

The initial product of transcription (the *primary transcript*) typically undergoes more or less extensive processing and/or modification to give the mature RNA product: see e.g. entries for mRNA, rRNA and tRNA.

Initiation and termination of transcription are important control points for gene expression: see e.g. ANTITERMINATION, CATABOLITE REPRESSION, OPERON. (See also POLAR MUTATION.)

transductant See TRANSDUCTION.

transduction The virus-mediated transfer of host DNA (chromosomal or plasmid) from one host cell (the *donor*) to another (the *recipient*). Transduction was first observed in bacteriophage/bacterium systems, but has since also been found to be mediated by certain viruses infecting eukaryotic cells (see RETROVIRIDAE). The account below concerns only phage/bacterium systems.

Essentially, when a phage replicates in a (donor) cell, a few progeny virions encapsidate pieces of host DNA instead of — or in addition to — phage DNA; these virions can adsorb to a new host cell and introduce their DNA in the usual way. There are two basic types of phage-mediated transduction.

(a) *Generalized transduction*: any of a wide range of donor genes may be transduced, and the transducing phage particles contain *only* donor DNA. In some systems any of the host genes has a more or less equal chance of being transduced, but in other systems some genes are transferred at higher frequencies than others. For example, in the BACTERIOPHAGE P22/*Salmonella* system, certain regions of the host chromosome resemble the P22 *pac* site, and packaging of chromosomal DNA may be initiated at and proceed from these sites [MGG (1982) *187* 516–518]; thus, the probability of a given gene being transduced depends on its distance from a *pac*-like site. (Certain 'high-frequency transduction' (HT) mutants of P22 have been shown to be defective in *pac* recognition [JMB (1982) *154* 551–563].)

The fate of the transduced DNA in the recipient cell (now called a *transductant*) depends on various factors. If the DNA is a complete replicon (e.g. a plasmid) it may be stably inherited by the transductant. If the DNA is a fragment of a chromosome or plasmid, it may undergo one of 3 possible fates. (i) It may be completely degraded by the recipient cell's RESTRICTION ENDONUCLEASE system. (ii) It may undergo RECOMBINATION with a homologous region of the recipient's chromosome (or plasmid), so that at least some of the genes it carries can be stably inherited (*complete transduction*). (iii) It may persist in the cell in a stable but non-replicating form (*abortive transduction*). (The transduced DNA in an abortive transductant may exist as a circular DNA–protein complex [Virol. (1980) *106* 30–40].) Any donor genes present (and linked to promoters) can be expressed, and (if they are dominant alleles) the transductant will express the donor phenotype in respect of these genes. However, when an abortive transductant divides, only one of the daughter cells will receive the donor fragment; the other may nevertheless receive sufficient donor-gene products to permit expression of the donor phenotype for one or a few generations. Such abortive transduction is normally manifest by the formation of minute (often microscopic) colonies on medium selective for transductants; only one cell in the colony actually contains donor DNA.

The transduction of one particular donor gene is a rare event (e.g. 10^{-7}–10^{-5}, depending e.g. on phage). If two or more genes are transduced together (*cotransduction*) they are assumed to occur on the same fragment of DNA and are thus closely linked in the donor; generalized transduction has thus been used in the detailed mapping of donor chromosomes, distances between markers being estimated by determining their cotransduction frequencies.

(b) *Specialized (restricted) transduction* is mediated only by temperate phages which integrate into the host chromosome (see LYSOGENY); only bacterial genes immediately adjacent to the prophage can be transduced, and the transduced DNA is covalently linked to some or all of the phage genes. For example, when a population of *Escherichia coli* cells lysogenized by BACTERIOPHAGE λ is induced, a small proportion of the progeny virions may carry either *gal* or *bio* host genes — often at the expense of certain phage genes at the opposite end of the prophage (virion component genes in *gal*-containing particles, control genes in *bio*-containing particles); such virions (specialized transducing particles, STPs) arise as a result of rare aberrant excision events in which recombination occurs between sites other than the

PROPHETIC PATENTS IN BIOTECHNOLOGY

While reading a technical article, you realize everyone has overlooked a clever twist, a way to better apply a new technology. You have no data to support your thinking, and no way to get the data without funding and lab time. What do you do? Assuming the rights are yours to barter, you risk giving away the idea if you tell potential collaborators. And most manufacturing companies will be reluctant to sign a confidentiality agreement until they know the basics of an idea. One option most scientists are unaware of is this: you may be able to protect your position by filing a "prophetic" patent.

Any good patent predicts the future, to an extent. An inventor who promptly files a patent application would be foolish to limit the patent coverage to the earliest data. Inventors must try to predict where a new development will lead, and they must include those educated guesses in their patent applications.

But there is a special class of patents based entirely on hypotheses, library research, and discussions—rather than lab work. These are truly prophetic patents. If the facts are right and the words are handled deftly, they can be as enforceable as any other patent.

Mechanics, Chemistry, or Pharmacy?

In the mechanical field, patents usually don't contain examples or experimental data. They contain drawings, and it's not too difficult to draw a machine or device that hasn't been built, if the inventor has a clear idea of how the parts should fit together. Besides, the actions of gears, levers, and other mechanical components are predictable. So it's not very difficult to obtain prophetic patents in the area of mechanical equipment. If a machine doesn't work as claimed, the patent simply becomes worthless.

Prophetic patents are more difficult to obtain in chemistry, because they could hinder research. If a patent could be obtained merely by making a new substance without having to prove its utility, anyone who later discovers a valid use for that substance could be prohibited from using it. That would let chemical companies lock up new areas of chemistry merely by synthesizing new compounds, and it would discourage research on those compounds by anyone else. (Stimulating research is an important public policy consideration behind the U.S. patent statute.)

In pharmacology, obtaining a prophetic patent is extremely difficult. There is a strong presumption that a minor change in molecular structure can generate unpredictable effects in living organisms. The amino acids offer a good example; two molecules with exactly the same chemical formula (such as tryptophan) can have totally different effects in living organisms—if one is the D isomer and the other is the L isomer. As another example, penicillin works well in mice, rats, and rabbits; no one would have predicted that it is relatively toxic to guinea pigs. So, if someone wants a patent on a new drug, they must prove the drug works as claimed. Tests on humans are not required; they can be done on lab animals[1] or even in tissue culture[2].

In any field, a prophetic patent application must satisfy several requirements. Every component or reagent must be known and available to the interested public. The necessary information must be in the application, or it must be widely known, rather than hidden away in an obscure publication[3]. The inventor must describe how to assemble the components into a workable system, in words or drawings that can be followed and used by people skilled in the art.

If those requirements can be met, the written description is called a *constructive* reduction to practice. In the words of the Patent and Trademark Office, "Patent law does not require an *actual* reduction to practice..."

Several court decisions state that a prophetic invention cannot be patented unless it is close enough to actual completion that only "routine experimentation" will then be required. That standard must be viewed in light of several other factors. First, there is no specific time limit on "routine experimentation." If an experiment takes months (e.g., if crop plants or large lab animals must grow and age to the point of senescence to test a plant growth regulator or a treatment for Alzheimer's disease), that's okay, as long as the experiments can be carried out by following instructions that don't require "undue creativity."

Second, the level of "ordinary skill in the art" can be very high. In biotechnology, the ordinary level of skill relates to scientists with Ph.D.s. And third, according to an important decision by the Court of Appeals for the Federal Circuit (CAFC)[4], a specific invention was unpatentable because it was "obvious"[5]. The patent in that particular case would have become a major force in the field of genetic engineering, but the inventors waited too long while various publications (including their own articles) advanced the state of knowledge in the field. In the words of the CAFC, "For obviousness under section 103, all that is required is a reasonable expectation of success." That is a powerful incentive for filing an initial application soon after reaching that stage. The initial application can be supplemented by "continuation-in-part" (CIP) applications with more data as the research progresses.

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The Strahilevitz Decision

Since the standards for prophetic patents in mechanics, chemistry, and pharmacy differ, an interesting set of opportunities (and problems) arises in chemical processing equipment. The leading court decision in this area is *In re Strahilevitz*[6].

Strahilevitz was a psychiatrist who worked with immunology and techniques for generating antibodies that bind to haptens. Haptens are too small to elicit an antibody response when injected into an animal. However, if haptens are bonded to large molecules such as bacterial proteins, an injected animal can generate antibodies that bind to the large molecule. Some of those antibodies will bind to the hapten region of the large antigen, and to hapten molecules alone.

It occurred to Strahilevitz that hapten-binding antibodies could be generated, purified, immobilized on a substrate such as tiny beads, and loaded into an affinity column. The column could be used to remove haptens from blood circulated through the column. This extra-corporeal device could, for example, remove molecules such as cocaine or heroin from the blood of intoxicated patients. Strahilevitz was worried that other people might be working on similar devices, so his attorney drafted a patent application and filed it promptly—before he had tested an actual working model.

The examiner rejected the patent application since it didn't have any data to prove that the device worked. The Court of Customs and Patent Appeals (which was later expanded into the CAFC) held that Strahilevitz was entitled to a patent because his application satisfied all the requirements of the patent law.

The patent issued in 1983 (#4,375,414), and the Strahilevitz decision has been cited about a half-dozen times with approval, and so is good law. Several related patents have also issued since then, and the area of research is still active, although it hasn't yet reached the commercial stage. One area of interest today involves using that technology to remove from blood low-density lipoproteins, to reduce cholesterol levels.

The Fleming Patent

Another example of a prophetic biotechnology patent is #4,666,425, "Device for Perfusing an Animal Head." A patent attorney who specializes in biochemistry and medical technology obtained it, using the pen name "Chet Fleming" (an unusual and non-recommended strategy, which was justified in that case based on the potential for trouble from animal rights advocates). Fleming knew about blood processing devices such as oxygenators, dialysis units, and slow-release drug delivery devices. With the help of neurosurgeons, he tracked down several old articles that reported experiments such as animal head transplants and isolated brain perfusions, some of which resulted in consciousness or brain waves after the operation. After figuring out a way to transfer the blood vessels in the neck to a blood processing device without disrupting blood supply to the head, Fleming filed a patent application on a mechanical blood processing system to perfuse a severed head.

Three points of strategy are worth noting: (1) The claims in his patent did not depend on whether the head regained consciousness. Fleming needed to describe only one valid use for the system, so he pointed out that studying a perfused head, even if the brain waves are flat, can be useful in the same way that studying a perfused kidney is useful. (2) He limited the claims to a mechanical device, without trying to claim chemical processes and without getting into interpretive words such as "alive." (3) Shortly before the patent issued, he filed a follow-up application covering chemical processes and a method of prolonging metabolic activities in a perfused head. The follow-up application is still pending, which is useful because he can work with it, amend the claims, and file additional copending applications. By contrast, the issued patent is a sitting target for opponents.

Fleming subsequently wrote[7] that his primary reason for obtaining the patent was to ensure that this line of potentially disturbing research is properly reviewed and controlled by institutional review panels. His patent is being reexamined, based on additional prior art discovered after the patent was published. As it turns out, similar experiments have been tried many times: transplanted dog heads regained consciousness for more than four days in the early 1970s, and isolated brains with no skulls or sensory organs generated brain waves "almost indefinitely" in the 1980s. So this line of technology is not as outlandish or unlikely as some people think.

Plasmid Recipes And The Lundak Decision

Prophetic patents can be an important strategy in genetic engineering, since they are sometimes used to avoid having to deposit a plasmid or culture of cells with a public depository. A scientist wanting to patent a new plasmid is obligated to divulge "the best mode of carrying out the invention" known on the day the application is filed. Some inventors will file an application a week or so before the plasmid becomes available, with a written recipe describing how to isolate the gene and make the plasmid. Since the plasmid wasn't available when the application was filed, the inventor had no obligation to deposit the plasmid itself. After the plasmid becomes available, the inventor can submit an affidavit saying, "My recipe worked just the way I predicted. Here's the data to prove it. Anyone skilled in the art can follow my recipe and make comparable plasmids without needing a culture of my cells."

That tactic is still being used, but it must be reconsidered in light of an alternate process. Under *In re Lundak* (227 USPQ 90, 1985) and the new federal rules (37 Code of Fed. Regulation 1.801-809), an inventor can refrain from depositing a biological material, and then argue that a deposit is unnecessary. If the examiner disagrees and demands that the material must be deposited, the material can be deposited at any time while the application is pending. However, this option exists *only if* the plasmids or cells existed and were specifically identified in the initial application; otherwise, the prohibition against adding new matter to an application would be violated. Therefore, the best strategy might be to file one application shortly before the material becomes available, and then file a second application as soon as the material is available. If the first application fails, the second can still succeed.

Definitional Problems

Without data showing that something can be done and actually has been accomplished, adequately defining crucial terms in the claims can be very difficult. For example, U.S. patent #4,751,081, issued in 1988 to Suslow and Jones and assigned to Advanced Genetic Sciences (Oakland, CA, now merged with DNA Plant Technology [DNAP]), describes how genes encoding chitinase were isolated, inserted into plasmids, and used to transform soil bacteria that colonize plant roots. Expressing chitinase in adequate concentration on the surfaces of plant roots might protect the plant roots from fungus or nematodes.

Claims 1–13 in Suslow's patent involve transformed soil bacteria. The prophetic part—and the problems—begin at Claim 14: "A method of inhibiting chitinase-sensitive plant pathogens..." The patent contains no data showing that soil bacteria with chitinase genes can inhibit nematodes, fungus, or any other plant pathogens. The data indicate that the chitinase gene did indeed transform soil bacteria, but nothing indicates that those cells could inhibit (or were even tested against) any type of plant pathogen.

The Suslow patent tried to use functional language to define the phrase "chitinase-sensitive plant pathogens;" they were defined as "fungi or nematodes which are inhibited, repelled or destroyed in the presence of chitinase." But that leaves a major question unanswered: How sensitive must they be, and to what concentration of chitinase, to fall within that phrase? If any quantity of chitinase is present around the roots of a plant, then according to the logic of Suslow's patent, it should exert some degree of inhibition, repulsion, or destruction of fungi or nematodes. Suppose that is tested and doesn't work the first time; it doesn't work at all unless a super-strong promoter is used. The failure of the first experiment would cast serious doubt on whether Suslow's functional language would survive a legal challenge. (If a patent claim covers non-functioning versions of an invention, the patent can be challenged and probably overturned on the ground that it is overbroad.) Sometimes, the only way to overcome that hurdle is by drafting more elaborate functional language. For example, a claim might include a phrase such as, "in concentrations which are expressed by transformed soil bacteria under competitive conditions."

One of the goals of functional language is to create a claim that effectively says, "If it works, then it's covered by my patent, and if it doesn't work, then it's not covered." However, the patent statute requires patents to contain "full, clear, concise, and exact terms" and to "distinctly claim the subject matter." If a court feels that a patent is overreaching or will do more harm than good by discouraging rather than promoting research, it can overturn nearly any prophetic patent which contains functional language, by declaring that the patent is "merely an invitation to experiment."

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United States Patent [19]

Grot et al.

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[54] PROTECTIVE CLOTHING OF FABRIC CONTAINING A LAYER OF HIGHLY FLUORINATED ION EXCHANGE POLYMER

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[*] Notice: The portion of the term of this patent subsequent to Sep. 4, 2001 has been disclaimed.

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Related U.S. Application Data

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[52] U.S. Cl. 428/286; 428/338; 428/421; 428/422

[58] Field of Search 428/421, 422, 248, 249, 428/246, 252, 262, 265, 286, 290

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[57] ABSTRACT

A protective garment fabricated at least in part from a composite fabric which contains a layer of a highly fluorinated ion exchange polymer having sulfonic acid functional groups, all the components of said composite fabric being hydrophilic.

21 Claims, No Drawings

PROTECTIVE CLOTHING OF FABRIC CONTAINING A LAYER OF HIGHLY FLUORINATED ION EXCHANGE POLYMER

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of our prior copending application U.S. Ser. No 06/168,638 filed July 11, 1980, now abandoned which in turn is a continuation-in-part of our prior application U.S. Ser. No. 06/138,681 filed April 9, 1980, now abandoned.

BACKGROUND OF THE INVENTION

Protective clothing of many types is now well-known for many and varied uses in protecting people from fire and harmful substances, such as suits for industrial workers, flame- and fire-resistant suits for firemen, forest fire fighters, race car drivers and airplane pilots, and suits for use by military personnel. Garments include not only complete, hermetic suits, but also individual garments such as trousers, jackets, gloves, boots, hats, head coverings, masks, etc.

Regulations restricting exposure to hazardous environments of various kinds, such as the Occupational Safety and Health Act, make it increasingly necessary to have better and more effective kinds of protective garments.

Such garments presently available are almost invariably of thick construction and heavy in weight, and are often fabricated at least in part from materials impermeable to water or water vapor, such as natural and synthetic rubbers and elastomers, chlorinated rubbers, etc. In the case of garments impermeable to water vapor, there is considerable discomfort to those wearing them, especially when the garments are of the hermetic variety, because of the entrapment of perspiration and body heat. Entrapment of heat and perspiration results in considerable discomfort of itself, and the heat stress which results from the prevention of loss of heat by the ordinary mechanism of evaporation of perspiration can rapidly reach a dangerous stage of heat prostration for the person wearing the garment.

It is an object of this invention to provide improved protective garments which possess the ability to permit the passage of water vapor through the fabric of the garment, and thereby provide improved comfort for the person wearing the garment.

It is another object of this invention to provide improved protective garments which possess not only the ability to permit the passage of water vapor through the fabric, but also the ability to act as a stable barrier to the passage of most organic substances, including toxic compounds, through the fabric. Such garments could protect those exposed to a wide variety of organic or harmful compounds.

It is a further object to provide such garments which are thin and light weight and which thus will more readily permit loss of heat by virtue of their light weight construction.

SUMMARY OF THE INVENTION

Briefly, the invention comprises using as a component of the fabric of a protective garment a layer of an ion exchange polymer, preferably a semipermeable ion exchange polymer. By "semipermeable" is meant permeable to water vapor but substantially impermeable to most organic substances.

More specifically, the present invention provides for the use in protective clothing of a composite fabric, said fabric containing as the essential component thereof a continuous film of a highly fluorinated ion exchange polymer having sulfonic acid functional groups, there being at least one fluorine atom attached to each carbon atom to which each said functional group is attached, said polymer having an equivalent weight no greater than about 2000, all the components of said composite fabric being hydrophilic.

There is also provided according to the invention a protective garment fabricated at least in part from the composite fabric described in the previous paragraph.

There is further provided according to the invention a process wherein (a) water permeates from a first space adjacent a first side of a barrier to a second space adjacent the second side of said barrier, said barrier having as the essential component thereof a continuous film of a highly fluorinated ion exchange polymer having sulfonic acid functional groups, there being at least one fluorine atom attached to each carbon atom to which each said sulfonic acid group is attached, said polymer having an equivalent weight no greater than about 2000, and (b) a hazardous substance, said substance being a toxic organophosphorus compound having a

moiety wherein R is a C_1 to C_{10} alkyl group, or a blistering agent which contains two or more chloroethyl groups, present in said second space (i) permeates only slowly into said barrier and (ii) that portion of said hazardous substance which permeates into said barrier is detoxified at least in part by said polymer, whereby the rate of penetration of said hazardous substance into said first space is substantially decreased.

DETAILED DESCRIPTION OF THE INVENTION

The composite fabric from which protective garments of the invention are made contains as the essential component thereof a continuous film or layer of a highly fluorinated ion exchange polymer having free sulfonic acid functional groups, there being at least one and preferably two fluorine atoms attached to the carbon atom to which the sulfonic group is attached. By "highly fluorinated" is meant that the polymer in ion exchange form has at least as many C—F groups as it has C—H groups.

A film of a highly fluorinated ion exchange polymer having free sulfonic acid groups swells markedly when it absorbs water, and thus may not be a preferred functional group when rejection of certain organic substances by the garment is required. However, the free sulfonic acid groups in such a polymer are easily converted to the alkali metal salt form by an aqueous solution of an alkali metal salt, which salt form of the polymer swells less. Conversion of the sulfonic acid form to the sodium salt form can gradually occur during wearing of a garment simply from contact with the salt in perspiration. When it is desirable to have a layer in a garment maintained in the sulfonic acid form even after

the wearer has perspired, as when there is potential exposure to a toxic or other hazardous substance which can be detoxified by contact with an acid, two separated layers of the highly fluorinated ion exchange polymer can be used; the inner layer of such polymer can substantially prevent contact of the salt in perspiration with the $-SO_3H$ groups in the second, outer layer of the polymer.

The highly fluorinated ion exchange polymers can be copolymers of fluorinated monomers containing the sulfonic functional group with nonfunctional monomers such as tetrafluoroethylene, trifluoroethylene, vinylidene fluoride, chlorotrifluoroethylene, etc. The polymers are preferably perfluorinated polymers prepared from perfluoro sulfonic monomers and tetrafluoroethylene. Such polymers and their preparation are now well-known in the art, and are described, e.g., in U.S. Pat. No. 3,282,875. Such polymers are unaffected by a large variety of chemicals including typical decontamination systems used after exposure of a protective garment to various toxic and harmful chemicals. Perfluorinated polymers of this type have retained good physical properties after exposure to chlorine gas and strong hot caustic solution within an operating chloralkali cell for times in excess of two years.

So as to have a high moisture permeability which will provide a garment having comfortable wearing properties, the highly fluorinated ion exchange polymer should have an equivalent weight of no greater than about 2000, preferably no greater than about 1500. (The equivalent weight of such a polymer is the number of grams of polymer which, when in H^+ form, provides one mol of hydrogen ion.) Equivalent weights as low as 1100 and even 1000 provide exceptionally high water vapor transmission rates. The water vapor transmission rates of fabrics containing a layer of such polymer is sufficiently high to permit the loss by permeation of enough perspiration so that a person wearing the garment is substantially more comfortable than he would be if weaving an impermeable garment. However, with increase in equivalent weight, the suppleness of the highly fluorinated ion exchange polymer increases, such polymer is more easily extruded in thinner films, and mechanical properties such as flex life improve; such factors can be considered when selecting the equivalent weight of the polymer to be used in any particular composite fabric.

The thickness of the layer of highly fluorinated ion exchange polymer is not critical to the permeation rate of water vapor, which is so high that it is almost independent of the thickness of the film in the range of thickness dealt with herein. In some cases where a garment is to protect the wearer from exposure to a harmful compound, extremely thin layers of the highly fluorinated ion exchange polymer may not be suitable. In those cases where the composite fabric is made by lamination of one or more component fabrics with a preformed film of the highly fluorinated ion exchange polymer or a precursor polymer thereof, the thickness of the film used is generally in the range of about 10 to 125 micrometers (about 0.4 to 5 mils), preferably about 10 to 50 micrometers. In those cases where one step in preparation of the composite fabric is coating a component fabric with a solution of the highly fluorinated ion exchange polymer or a precursor thereof followed by removal of the solvent by drying, composite fabrics containing a thinner layer of highly fluorinated ion exchange polymer, down to about 2.5 micrometers (0.1 mil) thick, and even down to about 1 micrometer (0.04 mil) thick, can be made. For garments intended for protecting the wearer from exposure to a harmful substance, the layer of highly fluorinated ion exchange polymer should be continuous, i.e., it should be substantially free of pinholes, so as to prevent leakage of organic substances to within the garment. A layer of highly fluorinated ion exchange polymer about 12 to 50 micrometers (0.5–2 mil) thick is most preferred.

The highly fluorinated ion exchange polymer should be of high enough molecular weight to be film forming and to have adequate toughness to survive conditions of wear without developing leaks which would destroy its integrity, and can be, e.g., linear or branched.

The component fabrics used in making the composite fabric are many and varied in type. They can be, but are not limited to, cotton, rayon, wool, silk, linen, polyester such as polyethylene terephthalate, polyamides such as polyhexamethylene adipamide, polyhexamethylene decanedicarboxamide, polyhexamethylene dodecanedicarboxamide, poly-epsilon-caproamide or the polyamide of bis-para-aminocyclohexylmethane and dodecanedicarboxylic acid, aramids such as poly-meta-phenylene isophthalamide or poly-para-phenylene terephthalamide, polyolefins such as polyethylene, polypropylene or polytetrafluoroethylene, acrylics such as polyacrylonitrile, polybenzimidazoles, polyarylene sulfides, polyarylene-imide-amides, polyphenol-formaldehyde, polyimides, glass, flame-retardant cotton, etc., and blends of two or more of the foregoing. Carbonized cotton, acrylic, etc., fiber or fabric, or other adsorptive materials in any form such as activated carbon, can also be included as components of the composite fabrics. A component fabric can be woven, including, e.g., plain and ripstop weaves, knitted, nonwoven, felted, spunbonded, or poromeric fabric, or a fibrillated film, or a film or extrudate made or treated by any means to make it porous or microporous. In the case of such microporous component, those having a pore size of at least about 0.5 micrometer are preferred. Activated carbon or other adsorptive substances can be incorporated in the composite fabric by distributing it in a thin foamed layer included as one component of the composite fabric, or in any one layer or between two layers of said ion exchange polymer, or in any other suitable manner.

All of the components of the composite fabric of the invention, whether they be fabrics or continuous films, should be hydrophilic in nature. The term "hydrophilic", when used in reference to a film, means that such film will transfer substantial amounts of water through the film by absorbing water on one side where the water vapor concentration is high, and desorbing or evaporating it on the opposite side where the water vapor concentration is low. The term "hydrophilic", when used in reference to a fabric, means that water will spread on the fabric and wick into its porous structure. In the case of those component fabrics listed in the previous paragraph which are not hydrophilic, such as microporous polytetrafluoroethylene fabric, they must be impregnated throughout the structure and on both surfaces with sufficient hydrophilic polymer to render them, in effect, reinforced hydrophilic films; non-hydrophilic materials when so impregnated and coated lose their non-hydrophilic character and behave as hydrophilic components. Films of the highly fluorinated ion exchange polymers referred to hereinabove are hydrophilic, and such polymers are suitable for render-

ing hydrophilic those component fabrics which would otherwise be non-hydrophilic.

The composite fabric can take any of manifold forms. In addition to the layer of highly fluorinated ion exchange polymer, the composite fabric further comprises at least one layer of component fabric, preferably at least two layers of component fabric which may be the same or different. When the composite fabric contains at least two layers of component fabric, preferably there will be at least one layer of component fabric on each side of the layer of ion exchange polymer so as to provide protection to the latter from mechanical damage. It is further preferred to use as one of the outermost component fabrics a layer of a flame-resistant and/or wear-resistant fabric, and to fabricate the garment with such component fabric being on the outside of the garment.

A preferred embodiment of the composite fabric is that made from only one layer of component fabric in addition to the layer of highly fluorinated ion exchange polymer. Such composite fabric is intended to be used in a protective garment with the layer of highly fluorinated ion exchange polymer on the outside of the garment, and the component fabric side of the composite fabric on the inside of the garment; this orientation of the composite fabric presents a smooth, non-porous, barrier surface against a cloud of toxic gas or liquid droplets, and thereby does not absorb or trap any of the toxic substance in pores or interstices of the composite fabric, thus permitting easy decontamination after exposure to the toxic substance. Garments which are fabricated with a porous or microporous surface toward the outside, once contaminated by entrapment of a toxic substance in the pores, are at least extremely difficult, and often impossible, to decontaminate, and when decontamination is impossible must be carefully disposed of after but a single use. The protective garments of the invention are easily decontaminated, and thus provide for multiple reuse of the garment. With the indicated orientation of the composite fabric, there is the further advantage that the inner layer of hydrophilic component fabric soaks up perspiration and brings it into direct contact with the outer layer of moisture-transporting ion exchange polymer. Accordingly, the composite fabric of the invention possesses advantages over known fabrics which have a hydrophobic microporous layer on either side of another component fabric.

It should be noted that there are some situations in which the exposed outer layer of highly fluorinated ion exchange polymer could be damaged, in which case the loss of integrity of the barrier layer of the garment would endanger the person wearing the garment; in those situations, it is advisable that a wear-resistant outergarment be worn over the protective garment to aid in precluding damage to the latter. Such overgarments, following contamination, can either be laundered for reuse, or be of inexpensive, light-weight construction adapted for discarding after exposure to a toxic substance.

The composite fabric can be made from the component fabrics and either a film of highly fluorinated ion exchange polymer or a fabric either melt- or solution-coated with a continuous layer of highly fluorinated ion exchange polymer. The composite fabric is made in some cases by the use of heat and either vacuum or pressure, and in other cases by using suitable adhesives or meltable or soluble polymers to adhere the several components together. In some cases, the highly fluorinated ion exchange polymer is maintained in the form of a melt-fabricable precursor, e.g., with functional groups such as $-SO_2F$, during formation of the composite fabric, and after the composite fabric has been made the melt-fabricable precursor is hydrolyzed or otherwise chemically modified to the ion exchange form defined above. In those cases where a precursor of a highly fluorinated ion exchange polymer having more difficultly hydrolyzable functional groups, such as $-SO_2F$ groups, is used in combination with a component fabric of polyolefin or polyfluorinated polyolefin, hydrolysis can be under any suitable conditions such as those used with hydrolysis bath A in the examples below, but when such a polymer is used in combination with a component fabric of a nylon, cotton, wool or other polymer which may be damaged by vigorous hydrolysis conditions, hydrolysis after fabrication of composite fabric prepared therefrom should be under milder conditions such as with ammonium hydroxide. A highly fluorinated ion exchange polymer having sulfonyl functionality can alternatively be put into the form of the sulfonic acid, sulfonamide or substituted sulfonamide, or an alkali metal, ammonium or amine salt thereof (preferred amines include p-toluidine and triethanolamine) before forming a composite fabric therefrom, and in such cases the composite fabric can be prepared by using a small amount of a highly fluorinated ion exchange polymer having, e.g., $-COOCH_3$ functional groups as an adhesive bonding agent, which can be hydrolyzed under mild conditions, or by using other types of adhesive such as ethylene/vinyl acetate based hot melt adhesives or two-component epoxy adhesives. Composite fabrics made without an adhesive bonding agent are preferred, inasmuch as most bonding agents interfere with passage of water through the composite fabric, and to the extent used, reduce the active area through which water permeates. If such an adhesive bonding agent is used, a highly fluorinated ion exchange polymer having, e.g., $-COOCH_3$ functional groups is preferred, as it can be hydrolyzed to alkali metal carboxylate form which has a high permeability to water; such polymers are known in the art, e.g., in Belgian Pat. No. 866,121. The various salt forms of a functional group can freely be interconverted from one to another, and to or from the free acid form, in either a component material or a composite fabric, as desired, by treatment with a solution containing the cation of the desired form. The composite fabric can be made from the components in some cases in a single operation, and in other cases by a series of sequential steps.

The composite fabrics described above can be used in fabrication of protective garments by techniques known in the art, including sealing of seams and joints by use of radio frequency heating or other known electronic bonding techniques, or by heat and pressure, in some cases with the aid of adhesives or sealants at the seams and joints to prevent leaks at those points. Garments can also be made by sewing, but in cases where a leak-free construction is desired the sewn seams should also contain a sealant or adhesive.

The composite fabrics and garments made therefrom are highly permeable to water vapor. Accordingly, a person wearing such a garment does not suffer heat stress which results from interruption of the usual mechanism of loss of body heat by evaporation of the water of perspiration, and discomfort from the retention of the water of perspiration within the garment is reduced. While the composite fabrics are also permeable to a few low molecular weight organic compounds such

as methanol and ethanol, and while the permeation rate for an organic compound depends on the type of compound and its molecular weight, the permeation rates for most organic compounds are extremely low and in the case of many organic compounds the composite fabric is substantially impermeable to the compound. It is believed that the composite fabrics described herein possess barrier properties against a variety of hazardous substances, poisonous compounds, blistering agents, lachrymators, and irritants. As will be seen, the composite fabrics permit the passage of large amounts of water vapor.

The protective garment of this invention is believed to have the ability to protect the wearer against hazardous substances, such as certain toxic organophosphorous compounds that are anticholinesterases, which compounds have the common feature that they contain a

moiety where R is a C_1 to C_{10} alkyl group, and halogenated organic sulfides and amines such as the blistering agents which contain two or more chloroethyl groups, e.g., compounds of the formula $(ClCH_2CH_2)_2Z$, where Z is S or NQ, and Q is CH_3—, C_2H_5— or $ClCH_2CH_2$—. The essential component of the composite fabric used in making the protective garment, a highly fluorinated polymer having —SO_3H functional groups and at least one fluorine atom attached to each carbon atom to which each —SO_3H group is attached, is a strong acid which is believed to be capable of detoxifying such hazardous substances. In the examples, it is demonstrated that highly fluorinated ion exchange polymer is capable of hydrolyzing triethyl phosphate, a compound chosen as a model compound to simulate the toxic organophosphorus compounds. The ability of the highly fluorinated ion exchange polymer to act as a barrier to such organic substances, and additionally to detoxify at least in part that portion which permeates into the barrier, thus substantially retards the rate of penetration of such organic substances into the space within a protective garment of the invention.

The composite fabrics have good mechanical properties, such as toughness, strength and flex life. Both the composite fabrics and garments fabricated from them have good storage stability, such that the garments can be retained for long periods before actual use of them.

To further illustrate the innovative aspects of the present invention, the following examples are provided.

In the examples, water permeabilities were measured in accordance with ASTM (American Society for Testing Materials) method E 96-66, using the upright or inverted cup techniques as indicated. Permeabilities to substances other than water were measured by a similar technique, except at uncontrolled, ambient relative humidity.

In Examples 1 and 2 apparatus for continuous preparation of composite fabric was employed which comprised a hollow roll with an internal heater and an internal vacuum source. The hollow roll contained a series of circumferential slots on its surface which allowed the internal vacuum source to draw component materials in the direction of the hollow roll. A curved stationary plate with a radiant heater faced the top surface of the hollow roll with a spacing of about 6 mm (¼ inch) between their two surfaces.

During a lamination run, porous release paper was used in contacting the hollow roll as a support material to prevent adherence of any component material to the roll surface and to allow vacuum to pull component materials in the direction of the hollow roll. Feed and takeoff means were provided for the component materials and product. In the feed means one idler roll of smaller diameter than the hollow roll was provided for release paper and component materials. The feed and takeoff means were positioned to allow component materials to pass around the hollow roll over a length of about 5/6 of its circumference. A further idler roll was provided for the release paper allowing its separation from the other materials. Takeoff means were provided for the release paper and a composite fabric.

EXAMPLE 1

A composite fabric was prepared from (1) a piece of component fabric having 27.5 threads/cm (70 threads/in) of 1.5 denier filaments of poly-meta-phenylene isophthalamide in the warp and 19 threads/cm (48 threads/in) of like filaments in the woof in a plain weave, having a weight of 15 mg/cm^2, about 10 cm by 15 cm, and (2) a piece of a film of a copolymer of perfluoro(3,6-dioxa-4-methyl-7-octenesulfonyl fluoride)(referred to herein as PSEPVE) and tetrafluoroethylene (referred to herein as TFE) having an equivalent weight of 1350, the film having a thickness of about 36 micrometers (1.4 mils) and being hydrolyzed on one surface only to a depth of about 15 micrometers (0.6 mil) to the —SO_3K form, the piece of film being slightly larger than the piece of fabric. The lamination was carried out in the apparatus just described, using a web of paper with a window cut in it to carry the components through the apparatus. The piece of fabric was taped into the window, and the piece of film was taped over the fabric. As measured by thermocouples, the hollow roll was heated to 240° C. by the internal heaters and the temperature indicated by a thermocouple at the radiant heaters was 360° C. The vacuum in the hollow roll was 0.84 Atmospheres below atmospheric pressure. The line was run at 30 cm/minute (1 ft/min), to provide a dwell time in the heated portion of the apparatus of 1.5 minutes. During lamination, the component cloth contacted the release paper on the heated hollow roll, and the film was placed with its unhydrolyzed side, i.e., the —SO_2F side, against the component fabric.

In the resulting composite fabric, the film was pulled deep into the surface contour of the fabric but not into the interior; the yarn crossover points of the fabric were not bonded together, and the composite fabric had a good hand.

The composite fabric was placed in a solution of 50 volume % methanol and 50 volume % of 28% aqueous ammonium hydroxide at ambient room temperature, about 18° C., for 45 hours, to hydrolyze the remaining —SO_2F groups. The composite fabric with sulfonic acid ammonium salt functional groups was then treated for 1 minute with 1N aqueous hydrochloric acid to put the functional groups into —SO_3H form, and part of that composite fabric was treated with aqueous NaCl solution to make the —SO_3Na form. The acid (hydrogen) and sodium salt forms were tested for water vapor permeability by the inverted cup method, with results as shown in Table 1.

TABLE 1

Form and orientation	Vapor transmission g/m² day
H form, component fabric facing water in the cup	25,570
Na form, component fabric facing water in the cup	28,780
Na form, component fabric facing outside the cup	7,300

EXAMPLE 2

A composite fabric was prepared from continuous lengths of (1) a component fabric of 40/2 cc yarns of a 50:50 blend of poly-meta-phenylene isophthalamide and poly-para-phenylene terephthalamide staple fibers woven in a 2 by 1 twill as described in Example 2 of U.S. Pat. No. 4,120,914, and (2) a film like that employed in Example 1 above. The lamination was carried out in the same apparatus just described, with the same conditions as in Example 1 except that the vacuum in the hollow roll was 0.675 Atmospheres below atmospheric pressure. As in Example 1, the component cloth contacted the release paper on the heated hollow roll, and the film was placed with its unhydrolyzed side against the component fabric. The composite fabric so made was found to be free of leaks. Half of the composite fabric so made was placed in a solution of 60% by volume of methanol and 40% by volume of 28% aqueous ammonium hydroxide at about 18° C. for 65 hours to hydrolyze the remaining $-SO_2F$ groups, washed with water, washed with an aqueous solution containing 2% by wt. acetic acid and 1% by wt. sodium chloride, washed with water, and air dried, the ion exchange groups of the resulting composite fabric being in the Na form. One sample of the resulting composite fabric was placed in boiling water for 30 minutes before testing for water permeability. Another sample of the same composite fabric was soaked in 2N hydrochloric acid to prepare the $-SO_3H$ form, washed with water, soaked in water at 60° C. for 20 minutes, and air dried. Samples were tested for water permeability, in all cases by the inverted cup method and with the component fabric side of the composite fabric facing the water in the cup, with the results shown in Table 2.

TABLE 2

Form	Vapor transmission g/m² day
Na form as first prepared	8,530
Na form after treatment in boiling water	28,470
H form	32,530

It should be noted that treatment of a highly fluorinated ion exchange polymer with water at high temperature, such as with boiling water, is known to cause the polymer to swell (see U.S. Pat. No. 3,684,747).

EXAMPLE 3

A composite fabric was prepared from continuous lengths of (1) a component fabric which was a 22 cut jersey having a weight of 15.6 mg/cm² (4.6 oz/sq yd) knit from 20/1 cc yarn of poly-meta-phenylene isophthalamide, and (2) a film like that employed in Example 1 above. The lamination was carried out with the same apparatus and in the same manner as described in Example 2, and the composite fabric so made was hydrolyzed in the same manner as in Example 2. The resulting hydrolyzed composite fabric felt softer than the composite fabrics of Examples 1 and 2, and had some stretch characteristics. A portion of the composite fabric was converted to hydrogen form by treating with aqueous hydrochloric acid, and another portion was converted to sodium form by treating with aqueous NaCl solution. Permeabilities to water were measured by the inverted cup technique, with the component fabric side of the composite fabric facing the water in the cup, with the results shown in Table 3.

TABLE 3

Form	Vapor transmission g/m² day
H	14,030
Na	16,030

EXAMPLE 4

A solution of 25 ml of triethyl phosphate, $(CH_3CH_2O)_3P{=}O$, in 75 ml of water was divided into two equal parts. To one part (A) was added 1.01 g of a PSEPVE/TFE copolymer which had been hydrolyzed to the form having $-SO_3H$ groups, having an equivalent weight of 1100, and in the form of a powder of 60 to 100 mesh. The other part (B) was retained as a control. Each part was separately stirred. Samples (5 ml each) of part A were taken at time intervals (stirring was temporarily stopped to permit the powder to settle when each sample was taken so that it would be free of powder), and the acid formed by hydrolysis was titrated with 0.1N NaOH solution using phenolphthalein indicator, as summarized in Table 4. Neither of two samples taken from part B, 5 ml after 88 hrs and 25 ml after 136 hrs, required any 0.1N NaOH for neutralization, thus showing that no hydrolysis occurred in the control part without the ion exchange catalyst.

TABLE 4

Time (hours)	ml. of 0.1 N NaOH
18	0.25
20	0.40
24	0.60
88	0.80
112	0.90

Based on the assumption that only monobasic hydrolysis of the ester occurred, the overall average rate of hydrolysis for the total 112 hours is about 0.02 meq. of phosphate ester group hydrolyzed per day per gram of ion exchange polymer. The rate for the initial 24 hour period was about 3 times greater.

EXAMPLE 5

Samples of PSEPVE/TFE film having an equivalent weight of 1075 and thickness of 127 micrometers were hydrolyzed to $-SO_3K$ form with a hydrolysis bath consisting of 15% by wt. potassium hydroxide, 25% by wt. dimethylsulfoxide and 60% by wt. water (referred to herein as hydrolysis bath A), the functional groups in one portion of the film were converted to $-SO_3Na$ form by soaking in a 10% by wt. aqueous solution of NaOH, in another portion of the film to $-SO_3Cs$ form similarly with an aqueous CsOH solution, and in yet another portion of the film to $-SO_3H$ form by treatment with aqueous hydrochloric acid. Permeabilities to various substances were determined as indicated in Table 4 by the inverted cup method.

TABLE 5

Metal ion of functional group	Compound	Vapor transmission, g/m² day
Na	methanol	29,000
Na	chloroform	5.9
Na	hexane	4.8
Na	carbon tetrachloride	5
Na	toluene	8.6
Na	$CFCl_2CF_2Cl$	6.3
Cs	methanol	612
Cs	carbon tetrachloride	0.8
H	hexane	1.65
H	toluene	6.25

EXAMPLE 6

Flame resistance tests

A composite fabric was prepared by heating under pressure a piece, 16 cm in diameter, of a microporous polytetrafluoroethylene cloth having an average pore size of 0.5 micrometers, a porosity of 80%, and a thickness of 25 micrometers (the cloth having a microstructure characterized by nodes interconnected by fibrils, made by high-rate stretching at an elevated temperature of an unsintered, dried paste extrudate of polytetrafluoroethylene, as described in U.S. Pat. No. 3,962,153, and commercially available from W. L. Gore & Associates, Inc., under the trademark Gore-Tex), and a piece, 10.5 cm in diameter, of a film of a PSEPVE/TFE copolymer having an equivalent weight of 1100 and a thickness of 25 micrometers (1 mil) in a hydraulic press at 240° C. for 1 minute and a force of 30,000 kg. The resulting transparent, leak free, composite fabric was treated with hydrolysis bath A at 100° C. for 1 hour, to put the functional groups of the copolymer in $-SO_3K$ form, washed and dried, and flame tested. In the flame test, a piece of the composite fabric, 12 cm by 4 cm, was held horizontally by metal clamps in the flame 3 cm above a burning wooden match for 15 seconds. The composite fabric did not burn; there was slight charring without destroying the fabric. The behavior was the same when the flame was applied either to the center or to the edge of the film.

In a second test, a piece of composite fabric prepared as described in the previous paragraph, except that the film of PSEPVE/TFE copolymer had an equivalent weight of 1200 and a thickness of 51 micrometers (2 mils), and the components were bonded at 290° C. with a force of 18,000 kg, was used. After hydrolysis with hydrolysis bath A as above, it was held vertically, and the flame of a propane torch was applied to the edge of the fabric. The fabric ignited only when the hot, inner, blue cone of the flame impinged on the fabric. The fabric was self-extinguishing, i.e., the fabric stopped burning when the flame was removed.

Although the flame resistance tests were carried out with composite fabrics wherein the functional groups of the copolymer were in the $-SO_3K$ form, the tests are nevertheless indicative of the flame resistance of the composite fabrics where the functional groups are in the $-SO_3H$ form.

Comparative Examples A, B, C and D

In Example A, permeabilities were measured for a film of regenerated cellulose (cellophane) having a thickness of 25 micrometers (1 mil). Although it has a high water vapor permeability, in excess of 10,000 g/m² day, and, by the inverted cup method had a permeability of 36 g/m² day to 2-chloroethyl ether and of 11 g/m² day to n-propyl sulfide, it shatters and tears when mechanically abused and it makes noise when flexed, and was thus considered unsuitable for use as a component of a composite fabric.

In Example B, a chlorinated polyethylene fabric (commercially available under the name "Chloropel") was found to have a permeability of 3000 g/m² day for 2-chloroethyl ether, which is so high as to make it unsuitable as a component of a composite fabric for protective garments. The material was also swollen and delaminated where contacted by 2-chloroethyl ether.

In Example C, a film 127 micrometers thick of a copolymer of ethylene and methacrylic acid having an equivalent weight of 576 was tested for permeability in both the free acid (hydrogen) form and in the sodium salt form. Although the permeability (inverted cup) to 2-chloroethyl ether was considered good, 8.8 g/m² day in the sodium form and 6.2 g/m² day in the hydrogen form, the permeability (inverted cup) to water vapor was 1690 g/m² day in the sodium form and 5 g/m² day in the hydrogen form, these values for water being considered too low to provide the comfort level desired for a protective garment.

In Example D, a membrane comprising a film of a polystyrenesulfonic acid having an ion exchange capacity of 2.7 meq/g of dry resin, backed by a fabric of vinyl chloride/acrylonitrile fibers having a weight of 14 mg/cm² and being 34% by wt. of the membrane, the membrane thickness being 0.6 mm (commercially available from Ionics, Inc.) was found to have a permeability (inverted cup) of 19,600 g/m² day for water vapor, and of 1,640 g/m² day for 2-chloroethyl ether.

Industrial Applicability

Composite fabrics containing a continuous film of a highly fluorinated ion exchange polymer as defined herein are useful in protective garments such as jackets, trousers, complete suits hermetically sealed, gloves, boots, hats, head coverings, masks, etc. The garments are broadly useful for providing protection to workers in the chemical industry, firemen, forest fire fighters, race car drivers, crop dusters and airplane pilots, and they may have value for defensive use by military personnel. They are believed to provide protection against blistering agents which contain chloroethyl groups and toxic organophosphorus compounds by a dual action of preventing penetration by part of the substance, and of detoxifying at least part of the substance which penetrates into the ion exchange barrier layer of the garment. The garments provided herein are technically advanced over those previously known in that they readily permit loss of perspiration and body heat while providing the needed protection. The garments are also waterproof in the sense that gross amounts of liquid will not penetrate the ion exchange film. The water entry pressure of the composite fabric is an order of magnitude above that of ordinary waterproof fabrics. Garments of the composite fabrics are virtually "watertight", yet "breathable". The composite fabrics can also be used for rain or water protection in any kind of rainwear, such as rainsuits, coats, parkas, ponchos, slickers, etc.

We claim:

1. Use in clothing to protect the wearer against hazardous substance of a composite fabric, said fabric containing as the essential component thereof a continuous

film of a highly fluorinated ion exchange polymer having sulfonic acid functional groups, there being at least one fluorine atom attached to each carbon atom to which each said functional group is attached, said polymer having an equivalent weight no greater than about 2000, all the components of said composite fabric being hydrophilic.

2. The use set forth in claim 1 wherein said polymer is a perfluorinated polymer.

3. The use set forth in claim 2 wherein said polymer has an equivalent weight no greater than about 1500, and the thickness of said film is in the range of about 2.5 to 125 micrometers.

4. The use set forth in claim 3 wherein the thickness of said film is in the range of about 10 to 50 micrometers.

5. The use set forth in claim 1 wherein said composite fabric further comprises a component fabric of fibers of poly-meta-phenylene isophthalamide or poly-para-phenylene terephthalamide or a blend thereof.

6. The use set forth in claim 1 wherein said composite fabric further comprises a component fabric of fibers of polyhexamethylene adipamide, polyhexamethylene decanedicarboxamide, polyhexamethylene dodecanedicarboxamide, poly-epsilon-caproamide or the polyamide of bis-para-aminocyclohexylmethane and dodecanedicarboxylic acid.

7. A garment to protect the wearer against hazardous substances fabricated at least in part from a composite fabric, said fabric containing as the essential component thereof a continuous film of a highly fluorinated ion exchange polymer having sulfonic acid functional groups, there being at least one fluorine atom attached to each carbon atom to which each said functional group is attached, said polymer having an equivalent weight no greater than about 2000, all the components of said composite fabric being hydrophilic.

8. The protective garment of claim 7 wherein said polymer is a perfluorinated polymer.

9. The protective garment of claim 8 wherein said polymer has an equivalent weight no greater than about 1500, and the thickness of said film is in the range of about 2.5 to 125 micrometers.

10. The protective garment of claim 9 wherein the thickness of said film is in the range of about 10 to 50 micrometers.

11. The protective garment of claim 7 wherein said composite fabric further comprises a component fabric of fibers of poly-meta-phenylene isophthalamide or poly-para-phenylene terephthalamide or a blend thereof.

12. The protective garment of claim 7 wherein said composite fabric further comprises a component fabric of fibers of polyhexamethylene adipamide, polyhexamethylene decanedicarboxamide, polyhexamethylene dodecanedicarboxamide, poly-epsilon-caproamide or the polyamide of bis-para-aminocyclohexylmethane and dodecanedicarboxylic acid.

13. The protective garment of claim 7, 9, 11 or 12 wherein said composite fabric consists of one layer of said continuous film and one layer of component fabric, and said garment is fabricated from said composite fabric so disposed that said film is toward the outside of said garment and said component fabric is toward the inside of said garment.

14. A process wherein (a) water permeates from a first space adjacent a first side of a barrier to a second space adjacent the second side of said barrier, said barrier having as the essential component thereof a continuous film of a highly fluorinated ion exchange polymer having sulfonic acid functional groups, there being at least one fluorine atom attached to each carbon atom to which each said sulfonic acid group is attached, said polymer having an equivalent weight no greater than about 2000, and (b) a hazardous substance, said substance being a toxic organophosphorus compound having a

moiety wherein R is a C_1 to C_{10} alkyl group, or a blistering agent which contains two or more chloroethyl groups, present in said second space (i) permeates only slowly into said barrier and (ii) that portion of said hazardous substance which permeates into said barrier is detoxified at least in part by said polymer, whereby the rate of penetration of said hazardous substance into said first space is substantially decreased.

15. The process of claim 14 wherein said barrier is in the form of a composite fabric.

16. The process of claim 15 wherein said polymer is a perfluorinated polymer.

17. The process of claim 16 wherein said polymer has an equivalent weight no greater than about 1500, and the thickness of said film is in the range of about 2.5 to 125 micrometers.

18. The process of claim 17 wherein the thickness of said film is in the range of about 10 to 50 micrometers.

19. The process of claim 18 wherein all the components of said composite fabric are hydrophilic.

20. The process of claim 15 wherein said composite fabric further comprises a component fabric of fibers of poly-meta-phenylene isophthalamide or poly-para-phenylene terephthalamide or a blend thereof.

21. The process of claim 15 wherein said composite fabric further comprises a component fabric of fibers of polyhexamethylene adipamide, polyhexamethylene decanedicarboxamide, polyhexamethylene dodecanedicarboxamide, poly-epsilon-caproamide or the polyamide of bis-para-aminocyclohexylmethane and dodecanedicarboxylic acid.

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Hawley's

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Dictionary

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organophosphorus compound. Any organic compound containing phosphorus as a constituent. These fall into several groups, chief of which are the following: (1) phospholipids, or phosphatides, which are widely distributed in nature in the form of lecithin, certain proteins, and nucleic acids; (2) esters of phosphinic and phosphonic acids, used as plasticizers, insecticides, resin modifiers, and flame retardants; (3) pyrophosphates, e.g., tetraethyl pyrophosphate, which are the basis for a broad group of cholinesterase inhibitors used as insecticides; (4) phosphoric esters of glycerol, glycol, sorbitol, etc., which are components of fertilizers. While many of these compounds play an important part in animal metabolism, those in group (3) are toxic and should be handled with extreme care.

organopolysilicate. See organoclay.

organosilane. See organosilicon.

organosilicon. An organic compound in which silicon is bonded to carbon (organosilane). Such compounds were first made by Friedel and Crafts in 1863. Silicon was found to have a remarkable chemical similarity to carbon, which it can replace in organic compounds. The silicon-carbon bond is about as strong as the carbon-carbon bond, and compounds containing them are similar in properties to all-carbon compounds. Organosilicon oxides (organosiloxanes or silicones) were discovered by F. S. Kipping in England in 1900; he found that Grignard reagents would react with silicon tetrachloride to form silicon-carbon-bonded polymers of both ring and chain types. These were named silicones because of the similarity of their empirical formula (R_2SiO) to that of ketones (R_2CO).

An organosilicon compound (tetramesityldisilene) containing a silicon-to-silicon double bond has been synthesized. It is a crystalline solid, mp 176C and has reactive properties similar to olefins. Compounds of this type are called silylenes.

See also silicone.

organosol. Colloidal dispersion of any insoluble material in an organic liquid; specifically the finely divided or colloidal dispersion of a synthetic resin in plasticizer, in which dispersion the volatile content exceeds 5% of the total.

See Plastisol.

organotin compounds. A family of alkyl tin compounds widely used as stabilizers for plastics, especially rigid vinyl polymers used as piping, construction aids, and cellular structures. Some have catalytic properties. They include butyl tin trichloride, dibutyltin oxide, etc., and various methyltin compounds. They are both [illegible]

Hazard: All are highly toxic, with a TLV of 0.1 mg/m³ of air.

See dibutyltin entries for specific data.

origanum oil. An essential oil used in pharmacy and as a flavoring.

"Orlon" [*Du Pont*]. TM for a copolymer containing at least 85% acrylonitrile. Available in various types of staple and tow.

Properties: Tensile strength (psi) 32,000–39,000, d 1.14–1.17, break elongation 20–28%, moisture regain 1.5% (21.2C, 65% RH), softens at 235C, soluble in butyrolactone (hot), dimethyl formamide (hot), ethylene carbonate (hot), resistant to mineral acids, fair to good resistance to weak alkalies. Insoluble in alcohol, acetone, benzene, carbon tetrachloride, and petroleum ether; soluble in dimethyl sulfoxide, maleic anhydride, ethylene carbonate, nitriles, and nitrophenols.

Hazard: Combustible, burns freely and rapidly.

Use: In apparel, usually blended with wool or other fibers.

Orn. Abbreviation for ornithine.

ornithine. (2,5-diaminovaleric acid).

CAS: 70-26-8. $NH_2(CH_2)_3CH(NH_2)COOH$.

A nonessential amino acid produced by the body and important in protein metabolism.

Properties: L(+)-ornithine: Crystals from alcohol-ether; mp 140C, soluble in water and alcohol. DL-ornithine: Crystals from water, slightly soluble in alcohol.

Derivation: Isolated from proteins after hydrolysis with alkali.

Use: Biochemical research; medicine.

ORNL. Abbreviation for Oak Ridge National Laboratory.

"Orotan" TV [*Rohm & Haas*]. TM for a synthetic tanning agent with attributes of vegetable tannins. Dark red, viscous solution; 31% tannin. Imparts high degree of tannage, strength, fullness, and solidity to leather. Solubilizing, penetrating, and bleaching agent.

orotic acid. (uracil-6-carboxylic acid; 6-carboxyuracil). CAS: 65-86-1.

$C_4N_2H_3(O)_2COOH$. Occurs in cow's milk and has also been isolated from certain strains of molds (*Neurospora*). A growth factor for certain microorganisms.

Properties: Crystals with mp 345–346C.

Use: Biochemical research, especially the biosynthesis of nucleic acids.

/ REVIEW

TRENDS IN THE DEVELOPMENT OF BACULOVIRUS EXPRESSION VECTORS

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We describe the current status and potential use of baculovirus vectors for the expression of foreign genes in insect cells. Trends in the development of transfer vectors for the expression of foreign genes under the control of the strong polyhedrin promoter of *Autographa californica* nuclear polyhedrosis virus and strategies for maximizing levels of expression are discussed. Baculovirus vectors have achieved widespread acceptance for their ability to express proteins of agricultural and medical importance. A baculovirus vector was used to express the first recombinant HIV envelope proteins to receive F.D.A. approval for clinical evaluation as a candidate vaccine for AIDS. These insect DNA virus vectors are contributing to understanding the molecular biology of gene and protein function and regulation in both vertebrate and insect systems.

A helper-independent baculovirus expression vector developed by Smith *et al.*[1] has been used for the expression of a wide variety of heterologous genes[2,3]. The baculovirus vector utilizes the highly expressed and regulated *Autographa californica* nuclear polyhedrosis virus (AcMNPV) polyhedrin promoter[1,2] modified for the insertion of foreign genes. This allows expression of prokaryotic or eukaryotic genes to produce fused or non-fused recombinant proteins. One of the major advantages of this invertebrate virus expression vector over bacterial, yeast, and mammalian expression systems is the very abundant expression of recombinant proteins, which are in many cases, antigenically, immunogenically, and functionally similar to their authentic counterparts (Table 1). In addition, baculoviruses are not pathogenic to vertebrates or plants and do not employ transformed cells or transforming elements as do the mammalian expression systems[6,7]. The baculovirus vector also utilizes many of the protein modification, processing, and transport systems that occur in higher eukaryotic cells and may be essential for the complete biological function of a recombinant protein.

Here, we briefly review the recent development and present status of baculovirus expression vectors. Apparent problems, known limitations, and opportunities are enumerated, and emerging trends for future applications are discussed. Emphasis is placed on the use of the baculovirus vector for expressing foreign genes at high levels and on the biological activity of recombinant proteins produced in insect cells. We also present and compare the sequences flanking the polyhedrin gene for those transfer vectors made in this laboratory that have been verified by DNA sequencing, as well as those that have been reported by other laboratories. Some errors in the literature concerning the derivation of certain transfer vectors are also clarified. The correct sequences for regions flanking the polyhedrin gene of all such plasmids are also reported.

THE BIOLOGY OF BACULOVIRUSES

Autographa californica nuclear polyhedrosis virus (AcMNPV), the prototype virus of the family *Baculoviridae*[8] has a wide host range and infects more than 30 species of Lepidopteran insects[9]. The genome of AcMNPV consists of double-stranded, circular, supercoiled DNA[10] approximately 128 kilobases (kb) in length[10,11]. During AcMNPV infection, two forms of viral progeny are produced: extracellular virus particles (ECV) and occluded virus particles (OV)[12]. The latter are embedded in proteinaceous viral occlusions, called polyhedra (polyhedron=singular). A polyhedrin protein with a molecular weight of 29,000 Daltons is the major viral-encoded structural protein of the viral occlusions[13]. In infected *Spodoptera frugiperda* cell cultures, polyhedrin accumulates to very high levels, routinely 1 mg/ml per $1.0–2.0 \times 10^6$ infected cells, accounting for at least 50% of the total "Coomassie stainable" protein of the cell detected on SDS-polyacrylamide gels[4].

The viral occlusions are an important part of the natural virus life cycle (summarized in Fig. 1), providing the means for horizontal transmission. When infected larvae die, millions of polyhedra are left in the decomposing tissue. The viral occlusions aid in protecting the embedded virus particles from environmental factors that would otherwise rapidly inactivate ECV. When larvae feed on contaminated plants, they ingest the polyhedra. The occlusions dissolve in the alkaline environment of the insect gut, releasing virus that invade and replicate in the cells of the midgut tissue. Secondary infection spreads to other insect tissues by the ECV form. Virus particles enter the cell by endocytosis or fusion, and the viral DNA is uncoated at the nuclear pore or in the nucleus. DNA replication occurs at about 6 hours post-infection (p.i.) and by 10 hours p.i. extracellular virus is released from the cell by budding. Polyhedrin protein can be detected by 12 hours p.i. but viral occlusions are not detected until 18–24 hours p.i. Extracellular virus levels reach a maximum between 36–48 hours p.i. but the polyhedrin protein continues to accumulate for 4–5 days until the infected cells lyse.

The polyhedrin gene of AcMNPV has been mapped and sequenced[5]. This gene has been shown to be nones-

TABLE 1 Foreign genes expressed by baculovirus vectors.

Gene	Source	Size (bp)	Protein (MW)	Biological Properties	Reference
AcMNPV polyhedrin	Genomic	1050	29K	Phosphorylated, Nuclear localization	59, 5
Bluetongue virus neutralization antigen VP2, VP3	cDNA cDNA	2800 2800	93K 92.5K	Antigenic, Immunogenic (VP2), Neutralization (VP3)	48, 60
Drosophila Krüppel gene product	cDNA	—	72K	Binds to ssDNA and dsDNA, Antigenic, Phosphorylated, Nuclear localization	28
E. coli chloramphenicol acetyl transferase	Genomic	785	27K	CAT activity, Antigenic	23
	Genomic	—	27K	CAT activity	30
E. coli β-galactosidase fusion protein	Genomic	9200	120K	β-galactosidase activity	29, 30
E. coli β-galactosidase	Genomic	3000	110K	β-galactosidase activity, Antigenic	4
Hepatitus B virus core antigen	cDNA	572	25/22K	Antigenic	23
Hepatitus B virus surface antigen	cDNA	1236	29/25K	Antigenic, Glycosylated, Assembled into lipoprotein particles	43
Human *c-myc* proto-oncogene	cDNA	—	64/61K	Antigenic, Phosphorylated, Nuclear localization	43 36
Human colony stimulating factor I	cDNA	—	34K	CSF-I activity, Antigenic, Glycosylated, Secreted, Dimeric assembly	R. Clark, per. com., 53
Human α-interferon	—	—	19.5K	Interferon activity, Antigenic, Signal peptide cleaved, Secreted	25, 31
Human β-interferon	Genomic	780	17/20.5K	Interferon activity, Antigenic, Glycosylated, Signal peptide cleaved, Secreted	2
HIV *env*	cDNA	2700	150/120–130/41K	Antigenic, Glycosylated, Proteolytic processing	46
HIV *env*	cDNA	—	160/120K	Antigenic, Immunogenic, Glycosylated	45
HIV *gag*	cDNA	1818	55/40K	Antigenic, Proteolytic processing	44
HIV *gag-pol*	cDNA	3114	24/55/40K	Antigenic, Proteolytic processing,	44
HTLV-I $p40^x$	cDNA	1750	40K	*Trans*-activation of HTLV-I LTR promoter, Antigenic, Phosphorylated, Nuclear localization	24
Human interleukin-2	cDNA	1000	16/15.5K	Interleukin-2 activity, Antigenic, Signal peptide cleaved, Secreted	17
Human parainfluenza virus type 3 hemmagglutinin-neuraminidase	cDNA	1716	70K	Hemagglutinating, Hemadsorption, Hemagglutination-inhibition activity, Antigenic, Immunogenic, Neutralization, Protection, Glycosylated, Cell surface	40
Influenza polymerase PA	cDNA	2200	87K	Antigenic	42
Influenza polymerase PB1	cDNA	2300	93K	Antigenic, PB1-PB2 complex formation	42
Influenza polymerase PB2	cDNA	2300	85K	Antigenic, PB1–PB2 complex formation	42
Influenza (fowl plague) hemagglutinin	cDNA	1750	—	Hemagglutinating, Hemadsorption, Hemolytic activity, Antigenic, Immunogenic, Neutralization, Protection, Glycosylated, Proteolytic cleavage, Cell surface	38
Influenza (A/PR/8/34) virus hemagglutinin	cDNA	—	65K	Hemagglutinating, Hemadsorption activity, Glycosylated, Proteolytic cleavage, Cell surface	32 33
LCMV glycoprotein precursor, GPC	cDNA	3300	72K	Antigenic, Glycosylated, Cell surface	18
LCM arenavirus (LCMV) nucleoprotein, N	cDNA	3300	62K	Antigenic	33 18, 33
N. crassa activator protein	Genomic	2900	100K	Site-specific DNA binding	37
Phaseolus vulgaris (French bean) phaseolin	cDNA	1400	51/45K	Antigenic, Glycosylated, Secreted	35
Polyoma virus T antigen	cDNA	—	100K	Origin-specific DNA binding, Antigenic	27
Pseudorabies virus gp50	—	—	46K	Antigenic, Immunogenic, Protection, Glycosylated (O-linked),	47
Punta Toro phlebovirus N	cDNA	1900	27K	Antigenic, Immunogenic, Neutralization	39
Punta Toro phlebovirus N_S	cDNA	1900	26K	Antigenic, Immunogenic	39
Simian rotavirus SA11 capsid antigen VP6	cDNA	1397	41K	Antigenic, Immunogenic, Oligomeric assembly	41
SV40 T, t antigens	Genomic	2702	19K	Antigenic, Correct small t splicing	22
Solanum tuberum (Potato) patatin	cDNA	1400	40K	Lipid acyl hydrolase, Acyl transferase activity, Antigenic	58

A dash (—) indicates data is unavailable or unknown. Antigenic refers to ability of the recombinant protein to be recognized by an antibody to the authentic protein by immunoprecipitation, immunoblot, immunofluorescence, and/or affinity column purification procedures. Immunogenic implies ability of the recombinant protein to raise an antibody in mice, rabbits or guinea pigs which will recognize the insect-derived protein and/or the authentic protein. Neutralization refers to the ability of antibodies raised to the insect-derived protein to neutralize virus in *in vitro* plaque reduction studies. Protection implies *in vivo* immunization with recombinant protein will provide protection to subsequent viral challenge.

sential for replication or for production of extracellular virus in cultured cells[1]. Deletion or insertional inactivation of the polyhedrin gene results in the production of occlusion negative viruses (Occ$^-$), which form plaques that are distinctly different from those of wild-type, occlusion positive (Occ$^+$) viruses. These distinctive plaque morphologies provide a way to visually screen for recombinant viruses in which the wild-type AcMNPV polyhedrin gene has been replaced with a hybrid gene of choice. The nonessential nature and high levels of expression of the polyhedrin gene and the ease with which recombinant Occ$^-$ viruses can be detected make the promoter of this gene particularly suitable for engineering as an expression vector.

Two comprehensive reviews presenting the biological properties and molecular biology of baculoviruses have recently appeared[14,15].

FACTORS AFFECTING EXPRESSION OF FOREIGN GENES BY BACULOVIRUS VECTORS

A wide variety of eukaryotic and prokaryotic genes have already been expressed successfully with baculovirus vectors in insect cells. A summary, giving the source of the gene, size of the protein, known post-translational modifications, and cellular location is shown in Table 1. The structural and functional similarity of recombinant proteins to authentic proteins is indicated by data from immunological, enzymatic, or biological assays. Recombinant proteins have been produced as fusion or nonfusion proteins at levels ranging from 1 to in excess of 500 mg/liter. Because the factors determining how well a foreign gene in this system is expressed are not yet well characterized, it is difficult to predict how efficiently different genes will be expressed. Many aspects of gene expression in this system are currently under study and new vectors are being designed and evaluated for enhanced levels of expression of specific gene products.

The regulation of late gene expression in baculoviruses is complex and not yet well characterized at the molecular level. Polyhedrin expression of wild-type AcMNPV can be variable and will differ with cells and tissues infected, and with the quality and quantity of cell culture media components. Polyhedrin expression is also not directly proportional to virus replication[16]. Polyhedrin accumulates to high levels in *Spodoptera frugiperda* cells infected with AcMNPV but is barely detectable in AcMNPV-infected *Bombyx mori* cells. Highest levels of expression require healthy *Spodoptera frugiperda* cells in which greater than 97% are viable and in exponential growth with a doubling time of 16–18 hours. Any significant alteration from the recommended cell culture conditions or procedures can result in significantly lower levels of expression for any polyhedrin-linked gene. Expression levels for different genes inserted into the same vector are often different and may be related to the length and nature of the leader sequence preceding the foreign gene. In certain cases, low levels of expression may be attributable to the gene product itself. This may be an intrinsic property of the protein or may be related to the pathways through which the protein is processed in the cell. Factors such as codon preference or RNA and protein stability have not been studied in any detail. In addition, different recombinant baculoviruses selected to contain a given foreign gene will often express that foreign gene at different levels. In all cases, it is desirable to isolate and characterize several recombinant baculoviruses from the same transfection mixture since not all recombination events with these vectors are equivalent.

Despite the large number of factors that influence late gene expression in baculoviruses, much of the present effort to maximize gene expression in this system has focused on localizing transcriptional and translational signals for the polyhedrin gene and the construction of improved transfer vectors. Because of the ease with which mutagenesis of transfer vectors can be carried out, most of the improvements in achieving high levels of expression at the molecular level in the near future are likely to be due to optimizing placement of the foreign gene within the transfer vector.

Construction of a baculovirus transfer vector. Most transfer vectors reported to date contain sequences from AcMNPV including the promoter of the polyhedrin gene and varying amounts of 5′ and 3′ viral DNA flanking the polyhedrin gene cloned into a high copy number bacterial plasmid. The desired foreign gene sequences in the recombinant plasmid can be transferred to wild-type AcMNPV by homologous recombination within a cell transfected with both the plasmid and wild-type virus DNAs (Fig. 2).

Table 2 AcMNPV transfer vectors

Plasmid Designation	Description or Construction	Reference
pAc101	(Fig. 4)	2
pAc311	(Fig. 4)	2
pAc960	(Fig. 4)	2
pAc373	(Fig. 3, 5, 6)	17, see text
pAc373-CAT	—	L. Guarino
pAc401	(Fig. 4), pAc311-*Bam*HI/*Kpn*I + *Bal*31 + *Sma*I linker	See text
pAc409	(Fig. 4), pAc311-*Bam*HI/*Kpn*I + *Bal*31 + *Sma*I linker	See text
pAc436	(Fig. 4), pAc311-*Bam*HI/*Kpn*I + *Bal*31 + *Sma*I linker	See text
pAc461	(Fig. 6), pAc311-*Bam*HI/*Kpn*I + *Bal*31 + *Sma*I linker	See text
pAc461-RI-(-)	pAc461-*Eco*RI + S1 nuclease	See text
pAc461-RI/PstI	pAc461-RI-(-)-*Sma*I + *Eco*RI-& *Pst*I linkers	See text
pAc461-PstI	pAc461-(-)-*Sma*I + *Pst*I linkers	See text
pAc510	—	See text
pAc610	(Fig. 6), pAc461-RI/PstI + MCS from M13mp10	See text
pAc611	(Fig. 6), pAc461-PstI + *Eco*RV/*Pst*I MCS fragment of pAc510	See text
pAc700	(Fig. 5), pAc373-*Bam*HI/*Kpn*I + oligos A1/A2	See text
pAc701	(Fig. 5), pAc373-*Bam*HI/*Kpn*I + oligos B1/B2	See text
pAc702	(Fig. 5), pAc373-*Bam*HI/*Kpn*I + oligos C1/C2	See text
pVL77	pUC19-*Xcy*I/Klenow + *Bam*HI-CAT-*Bam*HI/Klenow fragment of pAc373-CAT	See text
pVL101	(Fig. 4), pAc401-*Xcy*I + *Xcy*I-CAT-*Xcy*I fragment of pVL77	See text
pVL106	(Fig. 4), pAc436-*Xcy*I + *Xcy*I-CAT-*Xcy*I fragment of pVL77	See text
pAcC4	(Fig. 6)	E. Kawasaki, See text
pAcC5	(Fig. 6)	E. Kawasaki, See text
pAcYM1	(Fig. 6)	33
pAcRP6	(Fig. 6)	18
pAcRP18	(Fig. 6)	18
pEV55	(Fig. 6)	26

The first consideration in constructing a recombinant baculovirus is whether to clone a foreign gene sequence before or after the polyhedrin initiator codon, ATG, where the A is nucleotide +1. In most cases it is desirable to express a mature (nonfused) protein instead of a fusion product. Transfer vectors with unique cloning sites downstream from the polyhedrin ATG can be used to insert open reading frames into the polyhedrin coding sequence. The fusion products that result will contain amino acids from the N-terminal region of the polyhedrin protein as well as amino acids derived from translation of the leader sequence preceding the ATG of the foreign gene.

Currently, the most widely used transfer vector for introducing foreign genes into AcMNPV is pAc373[17] (Fig. 3). pAc373 was derived from a plasmid comprised of a 7 kb *Eco*RI fragment containing the AcMNPV polyhedrin gene cloned into the *Eco*RI-*Hin*dIII fragment of pUC8. As a result of *Bal*31 mutagenesis and addition of *Bam*HI linkers (CGGATCCG), pAc373 contains a deletion of the sequence between −8 (8 bases upstream from the polyhedrin ATG and approximately 40 bases downstream from the polyhedrin transcriptional start site) and the natural *Bam*HI sites at nucleotide +171. One unexpected result revealed by recent DNA sequencing across this region in this laboratory and by Matsuura *et al.*[18] is the presence of an additional 9 bases (CGAGATCCG) between the deletion ending at −8 and the sequences comprising the *Bam*HI site at position +171 (CGGATCC). Impurities in the linker preparation lacking *Bam*HI sites apparently account for these additional sequences and actually have a slightly beneficial effect on polyhedrin gene-linked expression over nearly equivalent vectors (such as pAcRP6) that lack them[18].

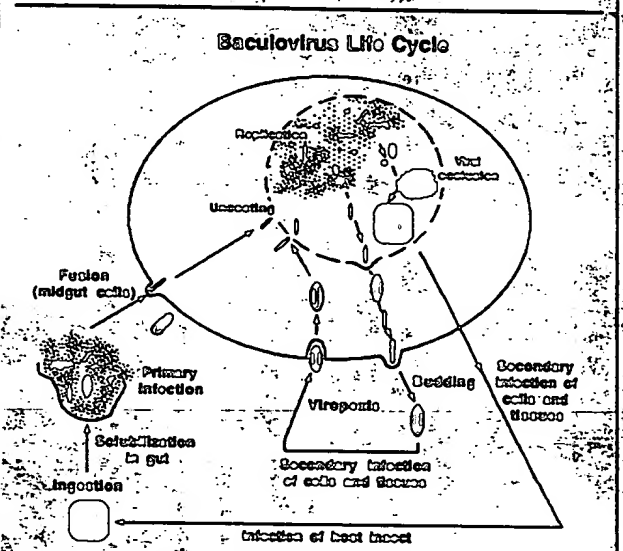


Fig. 1. Baculovirus life cycle. The schematic depicts the unique biphasic life cycle of a typical baculovirus. In the environment a susceptible insect ingests the viral occlusions from a food source. The crystal dissociates in the gut of the susceptible insect to release the infectious virus particles, which invade the gut cells, penetrate to the nucleus, and uncoat. Viral DNA replication is detected by 6 hours. By 10–12 hours post infection, extracellular virus buds from the surface to infect other cells and tissues. Late in infection (18–24 hours post infection) the polyhedrin protein assembles in the nucleus of the infected cell and virus particles become embedded in the proteinaceous occlusions. The viral occlusions accumulate to large numbers and the cells lyse. The viral occlusions are responsible for horizontal transmission among susceptible insects; the extracellular virus is responsible for secondary and cell to cell infection in cultured cells or the insect host. The polyhedrin gene is not essential for virus replication or infection in cultured cells.

Good expression of nonfused foreign proteins with pAc373 requires foreign genes that ideally have a short leader sequence. The lengths of the 5′ and 3′ noncoding sequences of genes that have been expressed have varied from 3 to 400 bases (Table 1). The significance of these sequences with respect to their effect on the levels of gene expression is unknown. Since the polyhedrin leader is very AT-rich, it is generally recommended that GC-rich or long 5′ leader sequences be trimmed as much as possible from the foreign gene prior to insertion into a transfer vector. All transfer vectors have the polyhedrin polyadenylation signal intact. Good levels of expression have been obtained using genes that have their own polyadenylation sites in addition to those of the polyhedrin gene.

All genes that expressed at high levels have been derived from cDNAs or genomic clones that do not contain introns. Although no intron-containing genes have been identified for AcMNPV[19–21], one recent report demonstrated correct and preferential utilization of the SV40 small t antigen splicing signals in a genomic fragment containing the genes coding for the large T and small t antigens[22]. The general applicability and usefulness of this approach for expression of other spliced genes have yet to be demonstrated.

SUMMARY OF AVAILABLE VECTORS

A variety of other transfer vectors suitable for production of fused (Figs. 4–5) or nonfused (Fig. 6) proteins have been constructed.

Vectors pAc401, pVL101, pAc436, pVL106, pAc409, pAc311, pAc360, and pAc101 (Fig. 4) and pAc700, pAc701, pAc702 (Fig. 5) were designed to facilitate production of fusion proteins. In each, a unique *Bam*HI or *Sma*I site is located within the polyhedrin coding sequence at different points downstream from the polyhedrin ATG start codon. DNA fragments cloned into one of these restriction sites will be expressed as protein fused to several N-terminal amino acids of the polyhedrin protein, provided that the reading frames are aligned. The number of polyhedrin amino acids added can vary from 1 to 58 and depends upon the fusion vector chosen.

Besides pAc373 (Figs. 3, 6), three additional transfer vectors (pAc461, pAc610, pAc611) have been characterized and used for production of nonfused proteins. In pAc461, polyhedrin sequences between −7 and +670 were deleted and replaced by one and one-half *Sma*I linkers (Fig. 6). The plasmids pAc610 and pAc611 were also constructed by inserting a multiple cloning site from M13mp10 into the *Sma*I site of pAc461 in either orientation (Fig. 6). Despite the usefulness of the polylinker in these vectors, recent data (described below) have shown that levels of expression of a variety of genes are higher in pAc373 or any of the fusion vectors than pAc461 or any of its derivatives[23].

The plasmids pAcC4 and pAcC5 are derivatives of pAc436 that contain an ATG start codon imbedded in a unique *Nco*I site, followed by a polylinker sequence. These vectors are useful for expressing genes that contain unique *Nco*I sites at their start codons (E. Kawasaki, Cetus Corporation, personal communication).

Miller *et al.*[26] reported a pEVIV vector in which a large portion of the polyhedrin gene was deleted in such a way as to create the sequence ATGGTACC at the initiating methionine. The unique *Kpn*I site adjacent to the ATG can be used to express proteins as fusions. A series of nonfusion vectors were developed by digestion of pEVIV with *Kpn*I followed by treatment with *Bal*31 to remove

varying numbers of nucleotides upstream from the ATG start codon. A polylinker containing *Bgl*II, *Xho*I, *Eco*RI, *Xba*I, *Cla*I, and *Kpn*I sites was then added. Rice *et al.*[27] used one of these vectors, pEV51, to express polyoma virus large T antigen. Another vector, pEV55 (Fig. 6), was recently used to express the *Drosophila* Krüppel gene product[28]. The entire polyhedrin leader is present in this vector and would be expected to provide higher levels of expression than pEV51, which has the polylinker inserted following position −22. Other vectors (described below) that leave the leader sequence intact, however, appear to express at much higher levels than pEV55.

Pennock *et al.*[29] described a vector, pGPB6874/*Sal*, that facilitates construction and selection of recombinant viruses carrying foreign genes under their own promoter control. A unique *Pst*I site is available for insertion of foreign genes that must provide their own promoter and polyadenylation sequences. The *E. coli* β-galactosidase gene under the control of the polyhedrin promoter is also present in this plasmid. The β-galactosidase gene is cloned into the natural *Bam*HI site of the polyhedrin gene at position +171 (similar to pAc101, Fig. 4), which is expressed as a 120K polyhedrin/β-galactosidase fusion protein. Recombinant viruses are identified as "blue plaques" by using the β-galactosidase indicator X-gal. Carbonell *et al.*[30] constructed a derivative of pGPB6874/*Sal*, pLC1, that contains the *E. coli* chloramphenicol acetyl transferase (CAT) gene fused to the promoter from the long terminal repeat in Rous sarcoma virus and inserted it into the AcMNPV genome as described above. Alleged[14] CAT activity was observed in *Spodoptera frugiperda*, *Aedes aegypti* (mosquito), and *Drosophila melanogaster* cell lines at very high multiplicities of infection, but not in *Mus musculus* (mouse) cell lines. In contrast, β-galactosidase was detected at high levels only in *S. frugiperda* cells, indicating that foreign gene expression in nonpermissive cells is promoter dependent and that late viral gene expression is restricted in such cells.

Maeda *et al.*[25] constructed a vector for transfer of foreign genes into *Bombyx mori* nuclear polyhedrosis virus (BmNPV). The plasmid p89B310 carries a 3 kb fragment containing the BmNPV polyhedrin gene promoter, a polylinker derived from pUC8, and a 3.1 kb fragment downstream of the polyhedrin gene. The polylinker, containing unique *Eco*RI, *Sma*I, *Sal*I, and *Pst*I sites, is located 18 bases upstream from the translational start of the polyhedrin gene. Recombinant BmNPV viruses containing a human α-interferon gene were used to infect silkworm larvae. Interferon was produced in large amounts and could be recovered from the hemolymph of these larvae. A 4-fold increase in the level of α-interferon was recently reported[31] with an improved transfer vector, pBM030.

Possee[32] and Matsuura *et al.*[33] described a variety of AcMNPV transfer vectors (pAcRP series, Fig. 6) with alterations in the polyhedrin leader sequence that were used to make recombinant viruses expressing the influenza virus hemagglutinin (HA) protein. Highest levels of expression were observed for vectors not deleted for more than 14 bases of the leader sequence just upstream from the polyhedrin ATG. Recombinants with smaller deletions (0, −7, −11) did not make more HA protein. Highest levels of expression for lymphocytic choriomeningitis virus (LCMV) genes were observed for recombinant viruses derived from the transfer vector pAcYM1 (Fig. 6), which has all of the upstream sequences of the polyhedrin gene including the A of the initiating ATG codon. Genes inserted into pAcYM1 are therefore expressed as nonfused proteins. Alterations within the 3′ coding sequences of the polyhedrin gene or sequences downstream from the polyhedrin gene did not influence the level of foreign gene expression.

Recent studies in this laboratory [23] have compared the levels of expression of chloramphenicol acetyl transferase, β-galactosidase, and tissue plasminogen activator from viruses constructed with a variety of fusion (pAc700, pAc701, pVL101, pVL106, pAc360, pAc311, pAc101) and nonfusion (pAc461, pAc373) transfer vectors. Highest levels of proteins and RNA were observed when portions of the amino terminus of the polyhedrin gene were fused in phase with the foreign genes. Intermediate or low levels of protein and RNA were observed for the nonfusion vectors tested. Although the level of RNA for each of the fusion derivatives was nearly equivalent to that of polyhedrin, protein levels were higher for derivatives in which the fusion was more than 30 bases downstream from the polyhedrin ATG. These results, and those of Matsuura *et al.*[33], suggest that the sequences just upstream from and possibly including the polyhedrin start codon are important for regulation of transcription. In addition, our results suggest that sequences important for regulation of translation extend downstream from the ATG codon as well. Based on these results, we have constructed and are currently characterizing in detail several new transfer vectors for production of nonfused products that provide very high levels of expression for certain gene products[34]. These new vectors are intended to replace pAc373, currently the most widely used AcMNPV transfer vector, as the vectors of choice for production of nonfused proteins under the control of the AcMNPV polyhedrin promoter.

BIOLOGICAL ACTIVITY OF RECOMBINANT PROTEINS

Recombinant proteins produced in insect cells with baculovirus vectors are biologically active and for the most part appear to undergo post-translational processing to produce recombinant products very similar to that of authentic proteins (Table 1). Recombinant proteins can be secreted[2,17,25,35]; targeted to the nucleus[24,28,36]; targeted to the cell surface[18,32,33,38–40]; assembled into oligomeric complexes[41–43] and disulfide-linked dimers (human colony stimulating factor I, R. Clark, personal communication); proteolytically cleaved[2,17,18,25,32,35,38,44,46]; phosphorylated[24,28,36]; N-glycosylated[2,18,33,35,38–41,45,46]; O-glycosylated[47]; myristylated (pp60$^{c\text{-}src}$, T. Roberts, personal communication); or palmitylated (SV40 Large T antigen, R. Lanford, personal communication). These products are in all cases antigenically, immunogenically, and functionally similar to their native counterparts (Table 1). Several viral antigens can neutralize virus *in vitro*[39,40,45,48] and provide protection to viral challenge *in vivo*[38,40,47].

Much remains to be learned about the nature of protein glycosylation in insect cells[49–51]. Glycosylation of foreign proteins produced in baculovirus-infected *Spodoptera frugiperda* cells has been examined only indirectly, by the incorporation of radiolabeled sugars, by assessment of sensitivities to endoglycosidases, or by the addition of the glycosylation inhibitor, tunicamycin[2,18,33,35,38–41,45,46]. The ability of insect cells to carry out N-glycosylation appears to involve the addition of high mannose-type oligosaccharides and, perhaps, some limiting triming of terminal sugars[52]. From studies of *Aedes aegypti* and *Aedes albopictus* (mosquito) cell lines, it is clear that N-linked oligosaccharides are deficient in sialic acid, galactose, and fucose[49]. Thus, the conversion of high-mannose to complex N-linked oligosaccharides does not appear to take place in those insect cells. In mosquito cells the absence of complex N-glycans has been confirmed by low levels of N-acetylglucosaminyl-, galactosyl-, and sialytransferases that are

responsible for the maturation of high mannose-N-glycans of mammalian glycoproteins[49]. Direct evidence for or against the presence of such enzymes in the *Spodoptera frugiperda* cell lines used for baculovirus propagation has yet to be demonstrated. It remains unclear whether this apparent or potential difference in the oligosaccharide processing pathways of insects and vertebrates will become an important factor in the production of mammalian glycoproteins in insect cells, or whether different insect cell lines will exhibit differing abilities to glycosylate foreign proteins.

Although the processing, transport, and targeting of the recombinant proteins produced in Lepidopteran insect cells appear to be similar to those in mammalian cells, much work remains to be done in elucidating the molecular details of such processes. It will be particularly important to discover the signals and mechanisms responsible for transport and targeting of insect proteins to the endoplasmic reticulum, Golgi, cell surface, nucleus, or other cellular organelles in order to predict the processing and localization of foreign proteins.

FUTURE DIRECTIONS

By all indications, the use of recombinant baculoviruses to express important human, animal, and plant proteins will continue to grow at a rapid rate. Improvements in the level of expression attributable to the optimal placement of the foreign gene within the transfer vector are likely to continue. Molecular dissection of the polyhedrin promoter is currently underway in several laboratories and is expected to reveal the important *cis*-acting transcriptional and translational signals flanking the polyhedrin gene and *trans*-acting factors responsible for the very abundant expression observed.

Much work remains to be done, however, at the molecular level in the area of protein processing. The majority of the work has been carried out with one baculovirus, AcMNPV, in only one cultured insect cell line derived from the ovarian tissue of *Spodoptera frugiperda*. Clearly much can be learned by studying the levels of expression and the protein processing, targeting, and transport of recombinant proteins in cell lines derived from other tissues and other susceptible host species.

Related baculoviruses with different host ranges should also be examined for suitability of growth in large scale cultures. The production of recombinant proteins in live insects has been suggested as an economical alternative to cell culture[51], but more work regarding the characteristics of proteins recovered from live insects remains.

The recent development of low-cost protein-free medium that supports insect cell growth, virus replication, and recombinant protein expression at levels equivalent to those in serum-containing medium[52], and methods for large-scale insect cell propagation in airlift fermentors[53] are important advances in the application of this technology on a commercial scale. These are just a few of the developments that can be expected to enhance the overall utility of the baculovirus expression vector system.

EXPERIMENTAL PROTOCOLS

Cells and virus. The wild-type baculovirus used throughout these procedures was the E2 strain of the *Autographa californica* nuclear polyhedrosis virus (AcMNPV)[10,11]. Standard procedures for insect cell culture and selection, screening, and propagation of wild-type and recombinant baculoviruses in *Spodoptera frugiperda* clone 9 (Sf9) cells have been described in comprehensive detail[4,23].

Recombinant plasmid constructs. Standard DNA techniques were used for the construction of recombinant plasmids[54].

The pAc400 series of vectors (pAc401, pAc409, pAc436, pAc461) were constructed by digesting pAc311[2] with *Bam*HI and *Kpn*I, and incubation with *Bal*31 exonuclease. The ends of the

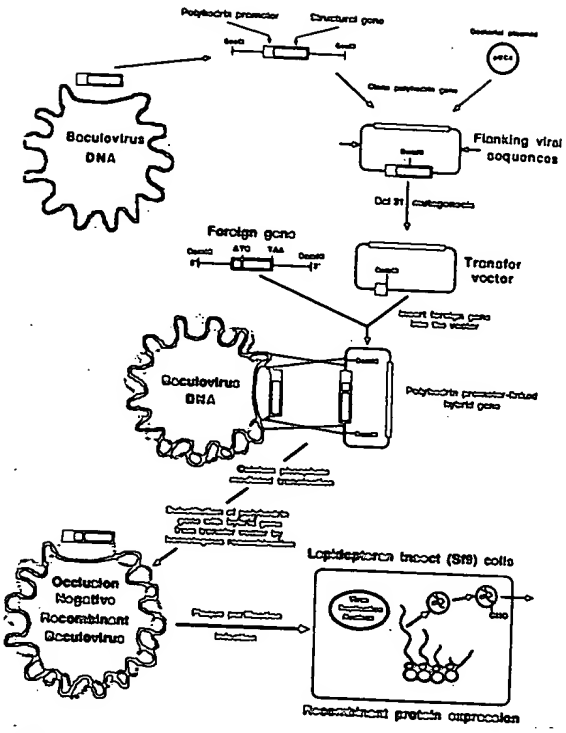


Figure 2 A schematic depicting the procedure and sequence for cloning a foreign gene and screening for recombinant plaques. An appropriate foreign gene fragment is inserted into a transfer vector in the proper orientation downstream of the polyhedrin promoter. Cultured insect cells are transfected with a mixture of viral and plasmid DNAs and the viral progeny are plated at 100–1000 plaques per plate. Occlusion minus viral recombinants are identified visually or by DNA hybridization and plaque-purified to homogeneity. The helper-independent recombinant baculovirus produces a recombinant protein within 48–72 hours post infection and the infection is essentially lytic within 4–5 days.

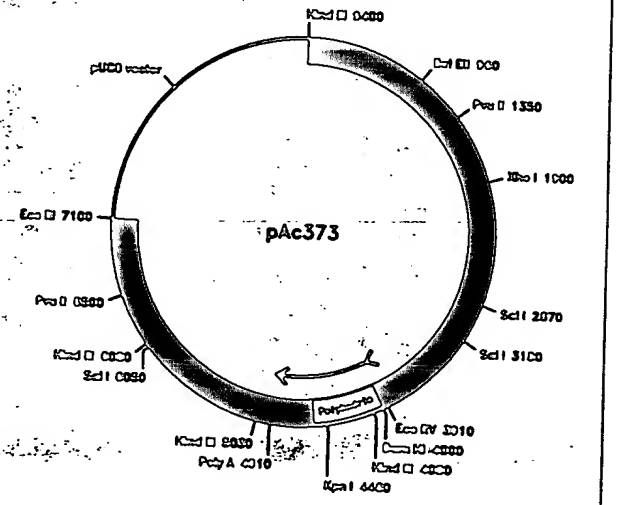
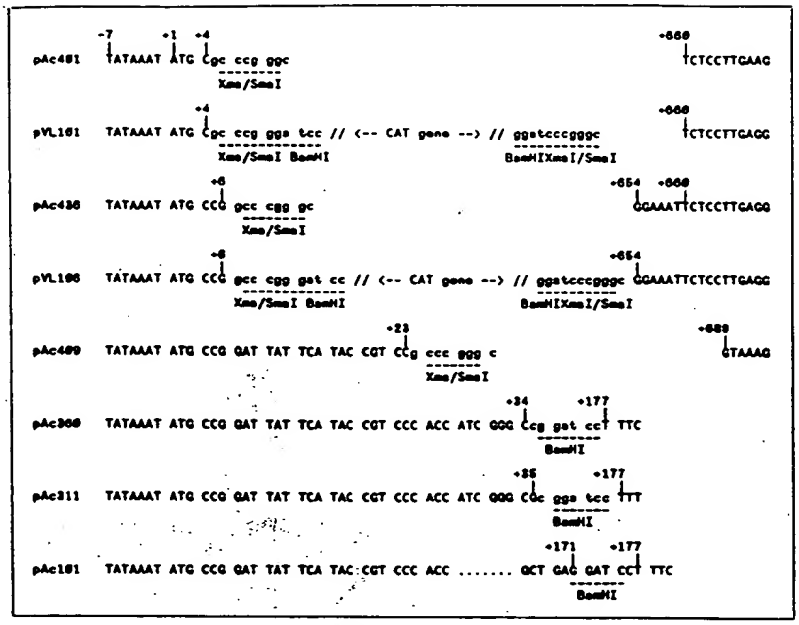


Figure 3 Restriction endonuclease map of the transfer vector pAc373. A unique *Bam*HI site is located following position −8 with respect to the translation initiation codon ATG of the polyhedrin gene. There are no cleavage sites for *Sma*I, *Pst*I, *Bgl*II, *Xba*I or *Sst*I. Good expression of nonfused foreign proteins requires foreign genes that ideally have a short leader sequence containing suitable translation initiation signals preceding an ATG start signal. The plasmid also contains the polyhedrin polyadenylation signal and the ampicillin-resistance (*amp*) gene and origin of replication for selection and propagation in *E. coli*.



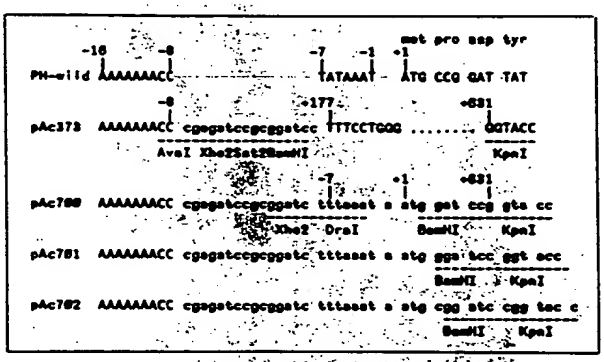


FIGURE 4 Nucleotide sequence spanning the polyhedrin start codon in the fusion vector derivatives pAc101, pAc311, pAc360, pAc401, pAc409, pAc436, pVL101, and pVL106. Sequences presented in lower case represent synthetic linkers used in the plasmid constructions which are not found in the native polyhedrin gene. Spaces are inserted to aid in visual alignment and dotted lines indicate sequences which are not shown. Underlining highlights sequences such as restriction endonuclease sites which differ among the transfer vectors. All of the sequences shown here, with the exception of the *Bam*HI and *Sma*I sites downstream of the CAT gene in pVL101 and pVL106, have been verified by sequencing across the unique restriction sites as described in the Experimental Protocol.

FIGURE 5 Nucleotide sequence spanning the polyhedrin start codon in the wild type AcMNPV virus, the transfer vector pAc373 for expression of nonfused foreign proteins, and the fusion vector derivatives pAc700, pAc701, and pAc702. Note the extra (unintentional) A preceding the ATG start codon and the (intentional) mutation at −7 to create a new *Dra*I site (TTTAAA) in the pAc700 series vectors, which are not found in the wild-type sequence.

DNA were then repaired with *E. coli* DNA polymerase (Klenow fragment). Phosphorylated *Sma*I (GCCCGGGC) linkers were added to the ends with T4 DNA ligase, the fragments were digested with *Sma*I, religated, and then used to transform *E. coli* JM83 cells. These plasmids have unique *Sma*I sites, and the left and right ends of the deletion in each plasmid are variable (Figs. 4 and 5).

The plasmids pVL101 and pVL106 are derivatives of pAc401 and pAc436 containing a 785 bp chloramphenicol acetyl transferase (CAT) gene[55]. The CAT gene was released from pAc373-CAT by cleavage with *Bam*H1, gel-purified, treated with Klenow, and ligated to pUC19[56] which had been digested with *Xcy*I (an isoschisomer of *Sma*I with 5′ overhanging ends) and treated with Klenow enzyme. One transformant, designated pVL77, has a *Sma*I/*Xcy*I site adjacent to a *Bam*HI site on both sides of the CAT gene. The plasmid pVL77 was cleaved with *Xcy*I, the CAT fragment gel-purified and ligated to *Xcy*I/CAP treated-pAc401 and pAc436, creating pVL101 (pAc401-CAT) and pVL106 (pAc436-CAT), respectively (Figure 4). The CAT gene is inserted in the same reading frame as the polyhedrin ATG start codon of pAc436 in pVL106, but it is out of phase in pVL101.

The plasmids pAc610 and pAc611 contain the multiple cloning site (MCS) of M13mp10 inserted in opposite orientations into the *Sma*I site of a derivative of pAc461 (Fig. 6). The plasmid pAc461 was digested with *Eco*RI and treated with S1 nuclease and T4 DNA ligase. The resultant plasmid, pAc461-RI-(−), has a deletion of about 1 kb of DNA around the unique *Eco*RI site of pAc461. The plasmid pAc461-RI-(−), was cleaved with *Sma*I, treated with CAP, and ligated to a mixture of *Eco*RI (CCGAATTCGG) and *Pst*I (CCTGCAGG) linkers. One of the resultant plasmids, pAc461-RI/PstI, which contained one of each type of linker, was cleaved with *Pst*I and *Eco*RI and ligated to the MCS from M13mp10. One clone containing a single copy of the MCS was designated pAc611. One pAc461-RI-(−) derivative that contained a single *Pst*I linker inserted into the *Sma*I site was designated pAc461-PstI. This plasmid was cleaved with *Eco*RV and *Pst*I and ligated to an *Eco*RV/*Pst*I fragment from pAc510, resulting in pAc610. The plasmid pAc510 was created in a manner similar to that described above for pAc611, except that it was derived from a pUC8 subclone of pAc461 that contains the 2.4 kb *Sal*I fragment spanning the polyhedrin gene. The plasmids pAc610 and pAc611 differ only in the orientation of the multiple cloning site.

The plasmids pAc700, pAc701, and pAc702 were constructed by ligating pairs of duplexed oligomers into pAc373 that was cleaved with *Bam*HI and *Kpn*I. To construct these plasmids,

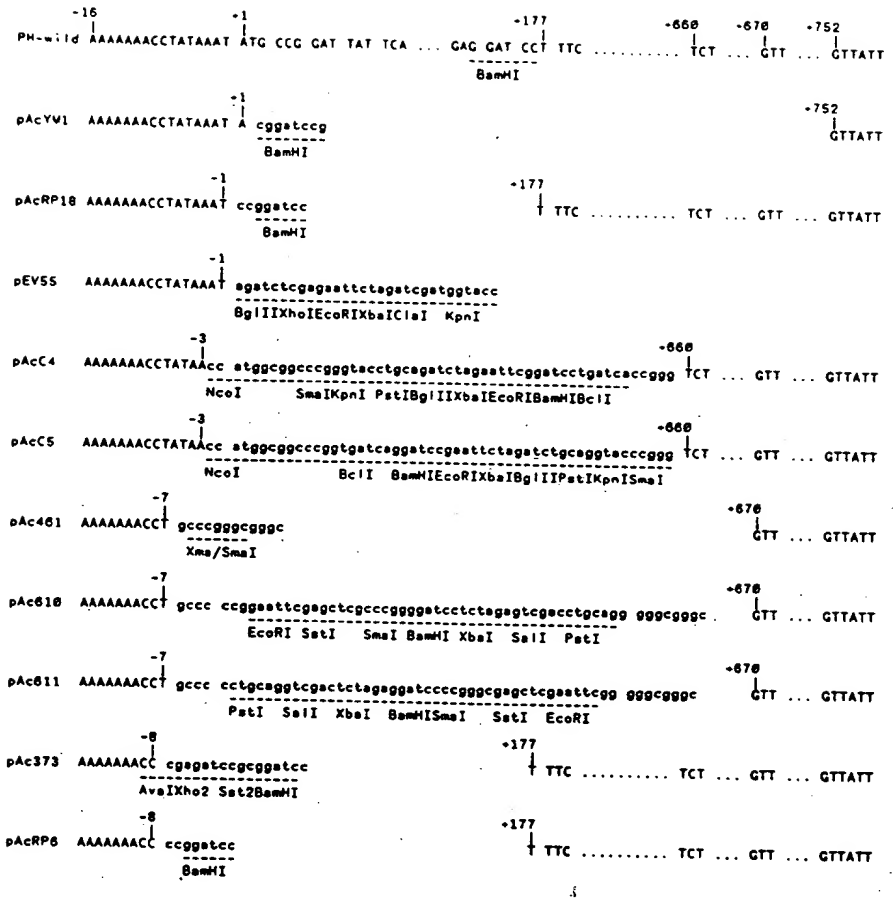


FIGURE 6 Nucleotide sequence spanning the polyhedrin start codon in the wild type AcMNPV virus and the transfer vectors pAc373, pAc461, pAc610, pAc611, pAcC4, pAcC5, pAcRP6, pAcRP18, pAcYM1, and pEV55 for expression of nonfused foreign proteins.

several small synthetic oligonucleotide fragments (A1/A2, B1/B2, and C1/C2) with the sequences

A1 5'-	GATCTTTAAATAATGGATCCGGTAC	-3'
A2 3'-	AAATTTATTACCTAGGC	-5'
B1 5'-	GATCTTTAAATAATGGGATCCGGTAC	-3'
B2 3'-	AAATTTATTACCCTAGGC	-5'
C1 5'-	GATCTTTAAATAATGCGGATCCGGTAC	-3'
C2 5'-	AAATTTATTACGCCTAGGC	-5'

were inserted into pAc373. The location of the *Bam*HI site is shifted so that it is just downstream of an ATG start codon. The plasmids pAc701 and pAc702 differ from pAc700 in that the *Bam*HI sites are shifted relative to the ATG by an additional 1 and 2 bases, respectively (Fig. 6). Note that a *Dra*I (TTTAAA) site created by a single base substitution and the insertion of an additional A preceding the ATG not found in the wild-type sequence was introduced into each of these vectors.

DNA sequencing. DNA fragments were subcloned into the vectors M13mp18 or M13mp19[56] and sequenced using the dideoxy chain termination method of Sanger *et al.*[57]. A 27-base oligonucleotide, TAMU4, (5'-CAATATATAGTTGCTGATAT-CATGGAG-3'), homologous to nucleotides -111 through -85 of the AcMNPV polyhedrin promoter, was synthesized and used as a sequencing primer. For other regions, a 17-mer M13 universal primer was used instead. The sequence was determined either on both strands or on the same strand of independently-isolated subclones.

Note added in proof: Expression of a polyhedrin-IGF-II fusion protein (Maruoka et al. 1987. J. Gen. Virol. 68:2599–2606), Hepatitis B surface antigen (Kang et al. 1987. J. Gen. Virol. 68:2607–2613), and HIV envelope protein (Rusche et al. 1987. Proc. Nat. Acad. Sci. USA. 84:6924–6928) were recently reported.

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STUDIES IN MICROBIOLOGY

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STUDIES IN MICROBIOLOGY VOLUME 2

Principles of Gene Manipulation

AN INTRODUCTION TO GENETIC ENGINEERING

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the intended recipient of cloned genes is transformable, RK290 alone is used. If the recipient is not transformable, the vector RK290 containing the foreign DNA insert first is transformed into an *E. coli* strain carrying pRK2013 and then conjugated into the desired recipient.

Olsen *et al.* (1982) discovered a small (2×10^6 mol. wt) multicopy, broad host range plasmid which had arisen spontaneously from the P group plasmid RP1. Presumably this plasmid retains the *ori*V and *trf*A* functions which appear to be the minimum requirements for replication of RK2. From this plasmid two derivatives were constructed. The first has two *Pst* I sites and can be used for cloning DNA where there is direct selection for the acquired trait, e.g. acquisition of antibiotic resistance or reversal of auxotrophy. The second plasmid contains an entire pBR322 molecule and if genes are inserted at the unique *Hind* III or *Bam* HI sites they can be detected by insertional inactivation of the Tc^R marker. Like pRK2501, these two vectors can be used only if the intended host is transformable.

Vectors derived from the broad host range group W plasmid Sa

Although a group W plasmid such as Sa (Fig. 8.3) can infect a wide range of Gram-negative bacteria, it has been developed

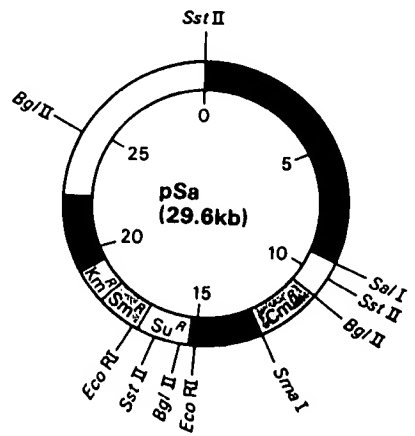

Fig. 8.3 The structure of plasmid pSa. The grey area encodes the functions essential for plasmid replication. The black areas represent the regions containing functions essential for self-transmission of pSa, the one between the *Sst* and *Sal* sites being responsible for suppression of tumour induction by *Agrobacterium tumefaciens*.

mainly as a vector for use with the oncogenic bacterium *Agrobacterium tumefaciens* (see p. 222). Two regions of the plasmid have been identified as involved in conjugal transfer of the plasmid and one of them has the unexpected property of suppressing oncogenicity by *A. tumefaciens* (Tait *et al.* 1982). Sufficient information for the replication of the plasmid in *E. coli* and *A. tumefaciens* is contained within a 4 kb DNA fragment. Leemans *et al.* (1982) have described four small (5.6–7.2 Mdal), multiply marked derivatives of plasmid Sa. The derivatives contain single target sites for a number of the common restriction endonucleases and at least one marker in each is subject to insertional inactivation. Although these Sa derivatives are non-conjugative they can be mobilized by other conjugative plasmids. Tait *et al.* (1983) also have constructed a set of broad host range vectors from plasmid Sa. The properties of their derivatives are similar to those of Leemans *et al.* (1982) but one of them also contains the bacteriophage λ *cos* sequence and hence is a cosmid.

A transposon as a broad host range vector

Transposons are mobile genetic elements which can insert at random into plasmids or the bacterial chromosome independently of the host cell recombination system. In addition to genes involved in transposition, transposons carry genes conferring new phenotypes on the host cell, e.g. antibiotic resistance. Grinter (1983) has devised a broad host range cloning vector based on transposon Tn7 which encodes Tp^R and Sm^R. Unlike the vectors just described, the transposon vector permits the cloned genes to be stably inserted into the chromosome. Such a vector could have useful industrial applications for it does not put an extra-genomic genetic load on the recipient cell.

Two compatible broad host range plasmids form the basis of the vector system. One of the plasmids is derived from RP1 but only encodes Tc^R. This plasmid is unstable and in the absence of selection for Tc^R is lost rapidly from host cells. A Tn7 derivative was inserted into this plasmid to generate pNJ5073. The Tn7 retains the original Tp^R and Sm^R markers but the DNA sequence which encodes transposition functions was replaced by a *Hind* III fragment containing the *E. coli trp*E gene. The second part of the vector system is plasmid pNJ9279 which encodes Km^R and is derived from plasmid R300b (≡ RSF1010, see p. 156). It is used to provide the transposition functions missing from the Tn7 derivative.

If pNJ5073 alone is introduced into a bacterium then, as noted above, in the absence of antibiotic selection it will not be maintained stably. Not even the Tp^R and Sm^R markers will be retained, for the variant Tn7 is unable to transpose. If pNJ9297 also is present then it can provide in *trans* the necessary transposition functions: selection of Tp^R and Sm^R followed by screening for Tc^S will identify those cells in which the defective transposon has hopped into the chromosome. In this way Grinter (1983) was able to select strains of *M. methylotrophus* and *P. aeruginosa* which had incorporated the *E. coli trp*E gene into their chromosome. In theory any gene can be inserted into the chromosome by replacing the *trp*E gene with an appropriate *Hind* III- generated DNA fragment.

As will be seen in Chapter 12 a formally analogous approach can be used to subjugate a eukaryotic (*Drosophila*) transposable element as a vector.

CLONING IN *B. SUBTILIS*

There are a number of reasons for cloning in *B. subtilis*. First, *Bacillus* spp. are Gram-positive and generally obligate aerobes compared with *E. coli* which is a Gram-negative facultative anaerobe. Thus the two groups of organisms may have quite different internal environments. Second, *Bacillus* spp. are able to sporulate and consequently are used as models for prokaryotic differentiation. The use of gene manipulation is facilitating these studies. Third, *Bacillus* spp. are widely used in the fermentation industry particularly for the production of exoenzymes. They can be tailored to secrete the products of cloned eukaryotic genes. Finally, from a biohazard point of view, *B. subtilis* is an extremely safe organism for it has no known pathogenic interactions with man or animals. Indeed it is consumed in large quantities in the East. Furthermore, in the literature between 1912 and 1983 there is only one authentic report of a human infection due to *B. subtilis* and that was in a severely compromised host—a drug addict.

Plasmid transformation in *B. subtilis*

An essential feature of any cloning experiment involving plasmids is transformation of a recipient cell with recombinant DNA. Although it is very easy to transform *B. subtilis* with fragments of chromosomal DNA there are problems associated with transformation by plasmid molecules. Ehrlich (1977) first reported that competent cultures of *B. subtilis* can be transformed with ccc plasmid DNA from *S. aureus* and that this plasmid DNA is capable of autonomous replication and expression in its new host. The development of competence for transformation by plasmid and chromosomal DNA follows a similar time course and in both cases transformation is first order with respect to DNA concentration suggesting that a single DNA molecule is sufficient for successful transformation (Contente & Dubnau 1979). However transformation of *B. subtilis* with plasmid DNA is very inefficient by comparison with chromosomal transformation for only one transformant is obtained per 10^3–10^4 plasmid molecules.

As will be seen later, much cloning in *B. subtilis* is done with bifunctional vectors that replicate in both *E. coli* and *B. subtilis*. Van Randen and Venema (1984) have shown that such bifunctional vectors can be transferred by replica-plating *E. coli* colonies containing them onto a lawn of competent *B. subtilis* cells. However plasmid transformation by replica plating differed in one respect from plasmid transformation in liquid. Whereas chromosomal integration of plasmid-borne chromosomal alleles with concomitant loss of plasmids occurred frequently during regular plasmid transformation of Rec^+ *B. subtilis*, this was a rare event during plasmid transfer by replica plating.

An explanation for the poor transformability of plasmid DNA molecules was provided by Canosi *et al.* (1978). They found that the specific activity of plasmid DNA in transformation of *B. subtilis* was dependent on the degree of oligomerization of the plasmid genome. Purified monomeric ccc forms of plasmids transform *B. subtilis* several orders of magnitude less efficiently than do unfractionated plasmid preparations or multimers. Furthermore, the low residual transforming activity of monomeric ccc DNA molecules can be attributed to low level contamination with multimers (Mottes *et al.* 1979). Using a recombinant plasmid capable of replication in both *E. coli* and *B. subtilis* (pHV14, see p. 170). Mottes *et al.* (1979) were able to show that plasmid transformation of *E. coli* occurs regardless of the degree of oligomerization, in contrast to the situation with *B. subtilis*. Oligomerization of linearized plasmid DNA by DNA ligase resulted in a subtantial increase of specific transforming activity when assayed with *B. subtilis* and caused a decrease when used to transform *E. coli*. An explanation of the molecular events in transformation which generate the requirement for oligomers has been presented by de Vos *et al.* (1981). Basically, the plasmids are cleaved into linear molecules upon contact with competent cells just as chromosomal DNA is cleaved during transformation of *Bacillus*. Once the linear

mice (Wagner *et al.* 1981) has resulted in abnormal expression (Lacey *et al.* 1983). In seven transgenic lines examined neither rabbit β-globin mRNA nor rabbit β-globin polypeptides were detected in the mouse erythroid cells. In two of the mouse lines, rabbit β-globin transcripts were found at low levels in particular but inappropriate tissues: skeletal muscle in one line, and testis in another. These patterns of transcription were heritable traits in the two lines, and Lacey *et al.* (1983) speculate that they are the result of DNA integration at abnormal chromosomal positions.

These results with a heterologous β-globin gene did not promise well for the application of transgenic mice to analysing gene regulation. However, subsequent experiments in which a rearranged mouse immunoglobulin K gene was introduced were extremely encouraging. In this case high expression of the K gene was restricted to the appropriate cell type, B lymphocytes, in several different transgenic mouse lines. It appeared that the microinjected K gene contained target sequences for gene activation which are specific for B lymphocytes and which can override the influence of different integration sites (Storb *et al.* 1984).

INTRODUCTION OF CLONED GENES INTO THE GERM LINE OF *DROSOPHILA* BY MICROINJECTION

P elements of *Drosophila*

P elements are transposable DNA elements which, in certain circumstances, can be highly mobile in the germ line of *Drosophila melanogaster*. The subjugation of these sequences as specialized vector molecules in *Drosophila* represents a landmark in modern *Drosophila* genetics. Through the use of P element vectors any DNA sequence can be introduced into the genome of the fly.

P elements are the primary cause of a syndrome of related genetic phenomena called P-M hybrid dysgenesis (Bingham *et al.* 1982, Rubin *et al.* 1982). Dysgenesis occurs when males of a P (paternally contributing) strain are mated with females of an M (maternally contributing) strain, but usually not when the reciprocal cross is made. The syndrome is confined mainly to effects of the germ line and includes a high rate of mutation, frequent chromosomal aberrations and, in extreme cases, failure to produce any gametes at all.

P strains contain multiple genetic elements, the P elements, which may be dispersed throughout the genome. These P

elements do not produce dysgenesis within P strains because transposition is repressed, probably due to the presence of a P-encoded repressor of a P element-specific *transposase* which is also encoded by the P element. However, when a sperm carrying chromosomes harbouring P elements fertilizes an egg of a strain that does not harbour P elements (i.e. an M strain), the P element transposase is temporarily derepressed owing to the absence of repressor. P element transposition occurs at a high frequency and this leads to the dysgenesis syndrome, the high rate of mutation results from the insertion into and consequent disruption of genetic loci.

Several members of the P transposable element family have been cloned and characterized (O'Hare & Rubin 1983). It appears that the prototype is a 2.9 kb element and that other members of the family have arisen by different internal deletion events within this DNA. The elements are characterized by a perfect 31 bp inverted terminal repeat. It is likely that this repeat is the site of action of the putative transposase. Three long open-reading frames have been identified in the prototype DNA sequences. These are candidates for transposase and repressor genes. Some of the short members of the family are defective. They cannot encode functional transposase but are transposable in *trans* in the presence of a non-defective P element within the same nucleus.

Spradling and Rubin (1982) have devised an approach for introducing the P element DNA into *Drosophila* chromosomes which mimics events taking place during a dysgenic cross. Essentially, a recombinant plasmid which consisted of a 2.9 kb P element together with some flanking *Drosophila* DNA sequences, cloned in the pBR322 vector, was microinjected into the posterior pole of embryos from an M-type strain. The embryos were injected at the syncytial blastoderm stage. This is a stage of insect development in which the cytoplasm of the multinucleate embryo has not yet become partitioned into individual cells (Fig. 12.3). The posterior pole was chosen because it is the site at which the cytoplasm is first partitioned, resulting in cells that will form the germ line. P element DNA introduced in this way became integrated into the genome of one or more posterior pole cells. Because of the multiplicity of such germ line precursor cells the integrated P element DNA was expected to be inherited by only some of the progeny of the resulting adult fly. Therefore the progeny of injected embryos were used to set up genetic lines which could be genetically tested for the presence of incorporated P elements.

A substantial proportion of progeny lines were indeed

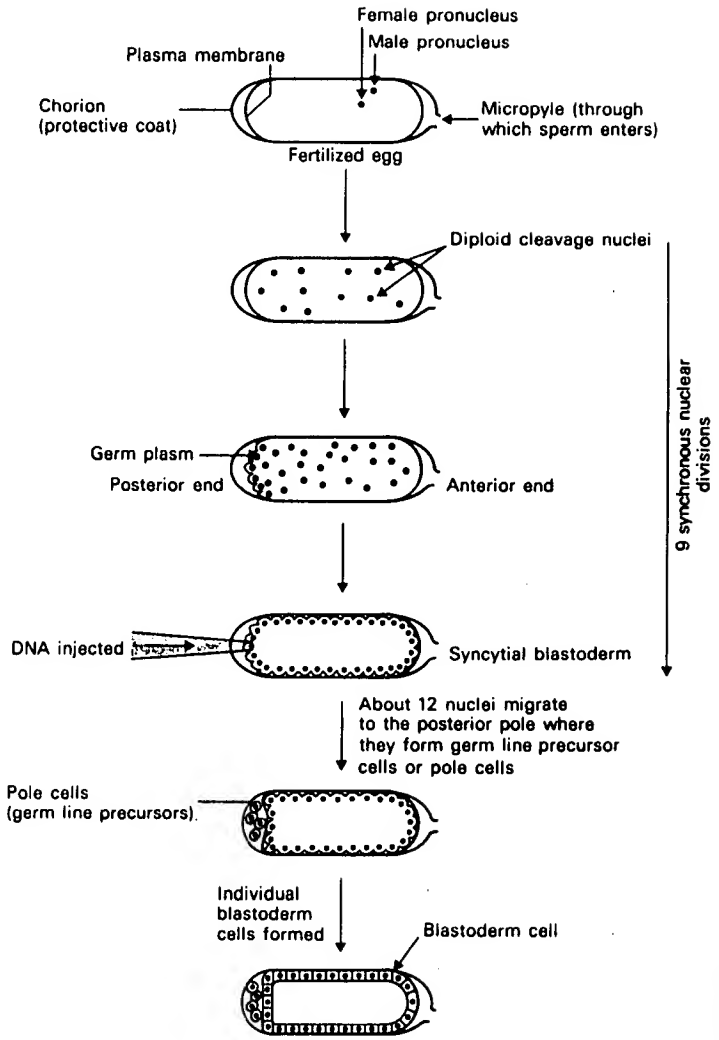

Fig. 12.3 Early embryogenesis of *Drosophila*. DNA injected at the posterior end of the embryo just prior to pole cell formation is incorporated into germ-line cells.

found to contain P elements integrated at a variety of sites in each of the five major chromosomal arms, as revealed by in-situ hybridization to polytene chromosomes. It may be asked whether integration really does mimic normal P element

transposition or whether it is simply some non-specific integration of the microinjected plasmid. The answer is that integration occurs by a mechanism analogous to transposition. By probing Southern blots of restricted DNA it was found that the integrated P element was not accompanied by the flanking *Drosophila* or pBR322 DNA sequences which were present in the recombinant plasmid that was microinjected (Spradling & Rubin 1982). Injected plasmid DNA must presumably have been expressed at some level *before* integration so as to provide transposase activity for integration by the transposition mechanism.

These experiments, therefore, showed that P elements can transpose with a high efficiency from injected plasmid into diverse sites in chromosomes of germ line cells. At least one of the integrated P elements in each progeny line remained functional as evidenced by the hypermutability it caused in subsequent crosses to M strain eggs.

P element as a vector

Rubin and Spradling (1982) exploited their finding that P elements can be artificially introduced in the *Drosophila* genome. A possible strategy for using the P element as a vector would be to attempt to identify a suitable site in the 2.9 kb P element sequence where insertion of foreign DNA could be made without disrupting genes essential for transposition. However, an alternative strategy was favoured. A recombinant plasmid was isolated which comprised a short (1.2 kb), internally deleted member of the P element family together with flanking *Drosophila* sequences, cloned in pBR322. This naturally defective P element cannot encode any of the putative protein products of the 2.9 kb prototype element (O'Hare & Rubin 1983). Target DNA was ligated into the defective P element. The aim was to integrate this recombinant P element into the germ line of injected embryos by providing transposase function in *trans*. Two approaches for doing this were tested. In one approach a plasmid carrying the recombinant P element was injected into embryos derived from a P–M dysgenic cross in which transposase activity was therefore expected to be high. This approach does have the disadvantage that frequent mutations and chromosomal aberrations would also be expected. In the other approach the plasmid carrying recombinant P element was co-injected with a plasmid carrying the non-defective 2.9 kb element. This approach is formally similar to the application of bacterial transposons discussed in Chapter 8.

In the first experiments of this kind embryos homozygous for a *rosy* mutation were microinjected with the P element vector containing a wild-type *rosy* gene. Both methods for providing complementing transposase were effective. Rosy$^+$ progeny, recognized by their wild-type eye colour, were obtained from 20% to 50% of injected embryos. The chromosomes of these flies contained one or two copies of the integrated *rosy*$^+$ DNA. Genetically stable progeny lines were established. In a simple extension of this scheme Rubin and Spradling (1982) demonstrated that any DNA, even if unselectable, could be introduced into the genome of *Drosophila* with a high efficiency.

Application of the P element vector

The initial successful demonstration of the P element vector system was rapidly followed by the simultaneous publication of three reports which described the reintroduction of three cloned *Drosophila* genes (Dopa decarboxylase, xanthine dehydrogenase and alcohol dehydrogenase) into the chromosomes of the fly. The genes were accompanied by substantial flanking sequences. These experiments were particularly exciting because in each case the regulation of the gene in question was correct.

Dopa decarboxylase is a gene which is subject to both temporal and tissue-specific regulation during development. Expression of the reintegrated copies of the gene showed the expected pattern of regulation (Scholnick *et al.* 1983). Xanthine dehydrogenase is the product of the *rosy* gene. Again, when the gene was reintroduced into the chromosomes of the fly, the tissue distribution of xanthine dehydrogenase was normal (Spradling & Rubin 1983). Finally, reintroduction of a cloned alcohol dehydrogenase gene resulted in normal expression according to several criteria: quantitative amounts of enzyme in larvae and adults; tissue specificity; and a previously recognized and characteristic developmental switch in transcription initiation site which is a feature of this gene, which has two sets of promoter elements (Goldberg *et al.* 1983).

The above successes with a variety of genes promise rapid progress in understanding DNA control signals which are required for the developmental and tissue-specific regulation of gene expression in *Drosophila*. A further aspect of gene regulation raised by these experiments concerns dosage compensation. *Drosophila* females are XX and males are XY, as in mammals. Both the fly and mammals face the problem of

controlling the level of expression of genes on their X chromosome since females have a double dose and the males a single dose, of each. In mammals this is achieved by total inactivation and heterochromatinization of almost all of an X-chromosome in each somatic cell of the female. *Drosophila* has a different mechanism; transcription of each copy of the X-linked genes in the female is less active than in the male. How this is brought about is not known. An insight is given by the finding that when the autosomal genes encoding dopa decarboxylase or xanthine dehydrogenase were integrated into the X-chromosome, expression was at least partially dosage compensated. This should be amenable to further analysis using the P element vector.

A final example of the application of this vector involves the heat shock response of *Drosophila*. High temperatures and other stress-inducing treatments evoke a dramatic change in the pattern of gene expression in *Drosophila*. Among many effects, transcription of certain genes, the heat shock genes, is greatly increased. This can be examined in many ways (see, for example, Bienz & Pelham 1982) but is most striking in the polytene chromosomes where the heat shock rapidly induces large puffs. Lis *et al.* (1983) have constructed a hybrid gene comprising the 5′ flanking region of a *Drosophila* heat shock gene and some accompanying amino-terminal heat shock protein codons, fused in phase with the *E. coli* β-galactosidase gene. The fused gene and a selectable *rosy*$^+$ gene were cloned into the P element vector and then inserted into the *Drosophila* genome. Three lines of flies were established, two contained a single inserted DNA and one contained two copies of the DNA at separate sites. On heat shocking such flies large chromosomal puffs occurred in the polytene chromosomes at the sites of insertion. By dissecting out organs of the larvae and adult flies and then incubating them in the chromogenic β-galactosidase substrate, X-gal, it was evident that β-galactosidase activity was inducible by heat shock and showed the expected widespread distribution throughout the animal's tissues.

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Parathion Hydrolase Specified by the *Flavobacterium opd* Gene: Relationship between the Gene and Protein

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The sequence of a 1,693-base-pair plasmid DNA fragment from *Flavobacterium* sp. strain ATCC 27551 containing the parathion hydrolase gene (*opd*) was determined. Within this sequence, there is only one open reading frame large enough to encode the 35,000-dalton membrane-associated hydrolase protein purified from *Flavobacterium* extracts. Amino-terminal sequence analysis of the purified *Flavobacterium* hydrolase demonstrated that serine is the amino-terminal residue of the hydrolase protein. The amino-terminal serine corresponds to a TCG codon located 87 base pairs downstream of the presumptive ATG initiation codon in the nucleotide sequence. The amino acid composition of the purified protein agrees well with that predicted from the nucleotide sequence, using serine as the amino-terminal residue. These data suggest that the parathion hydrolase protein is processed at its amino terminus in *Flavobacterium* sp. Construction in *Escherichia coli* of a *lacZ-opd* gene fusion in which the first 33 amino-terminal residues of *opd* were replaced by the first 5 residues of *lacZ* resulted in the production of an active hydrolase identical in molecular mass to the hydrolase isolated from *Flavobacterium* sp. *E. coli* cells containing the *lacZ-opd* fusion showed higher levels of hydrolase activity than did cells containing the parent plasmid.

The microbial degradation of hazardous waste offers a promising strategy by which such some wastes may be economically and safely detoxified. For selected situations, microbial processes have considerable advantages over other technologies in that microbial processes can yield precise products, function at low concentrations of solute, and require relatively low levels of technology for construction and maintenance. However, there are relatively few instances in which microbial processes are being actively used to control hazardous wastes.

Organophosphate compounds such as the insecticide parathion (*O,O*-diethyl-*O*-4-nitrophenyl phosphorothioate) are susceptible to microbial hydrolysis by bacterial parathion hydrolases. In fact, such soil hydrolases are thought to play a role in the relatively low persistence of these compounds. Since organophosphates constitute a large fraction of the insecticides used in the industrialized countries, there is a need for economical and reliable methods to detoxify organophosphate wastes (such as residual pesticide concentrates in their original containers, contaminated stock solutions, and dilute pesticide solutions resulting from the washing of spraying equipment). This need has stimulated recent research on parathion hydrolases.

Parathion hydrolase activities have been described in a variety of bacterial isolates and are characterized by broad substrate ranges for compounds structurally related to parathion, broad temperature and pH optima, and high stability (1, 10, 13, 14). Of the parathion hydrolases that have been characterized in detail, the enzymes encoded by the related *opd* genes of *Flavobacterium* sp. strain ATCC 27551 and *Pseudomonas diminuta* MG are noteworthy because of their high specific activities (10, 16). The hydrolase-producing *Flavobacterium* strain has been used in a pilot-scale system to detoxify waste containing high concentrations of the organophosphate insecticide coumaphos [*O,O*-diethyl-*O*-(3-chloro-4-methyl-2-oxo-2H-1-benzopyran-7-yl) phosphorothioate] (6).

Our laboratory has been particularly interested in modifying the *Flavobacterium opd* gene in order to maximize parathion hydrolase activity in other bacterial hosts. Our specific goals in this study were to (i) determine the nucleotide sequence of the *Flavobacterium opd* gene; (ii) determine the amino-terminal sequence and total amino acid composition of the hydrolase protein isolated from *Flavobacterium* extracts; and (iii) characterize the hydrolase proteins produced in *Escherichia coli* strains when the *opd* gene (and mutagenized *opd* genes) is activated by an *E. coli* promoter. While this work was in progress, the nucleotide sequences of the *Flavobacterium* and *P. diminuta opd* genes were reported (4, 9). We discuss the differences between our nucleotide sequence and those sequences.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Flavobacterium* sp. strain ATCC 27551 and the conditions for its culture have been described previously (10, 17). *E. coli* DH5α (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was used as the host for plasmids pWM513 and pUC19 (described below) for hydrolase purification. pWM44 is a hybrid plasmid in which a 7.3-kilobase-pair (kb) *Pst*I fragment from the *Flavobacterium* plasmid pPDL2 was inserted into pBR325 (11). pWM513 is a subclone of pWM44 in which a 1.3-kb *Pst*I fragment containing the *opd* gene was inserted into pUC19 (21).

DNA sequencing. The sequencing strategy is shown in Fig. 1. Subfragments of pWM44 and pWM513 were ligated into M13 vectors mp18 and mp19 (21) and sequenced by the dideoxy method of Sanger et al. (15), using a Sequenase kit (U.S. Biochemical Corp., Cleveland, Ohio) and a Macrophore sequencing apparatus (LKB Instruments, Inc., Rockville, Md.). The DNA sequences of several subfragments were independently determined by using a model 370A sequencer (Applied Biosystems, Inc., Foster City, Calif.),

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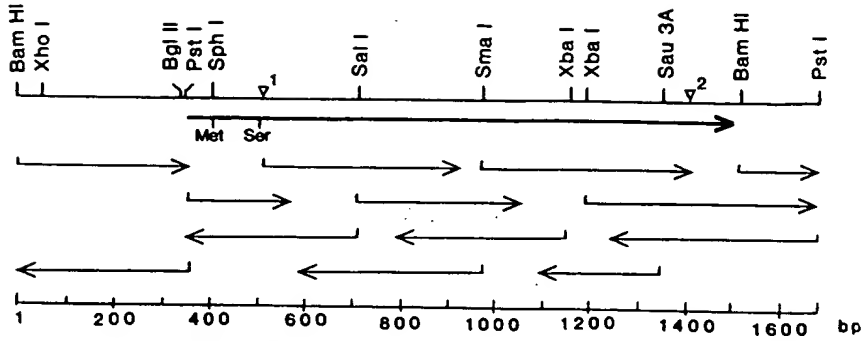

FIG. 1. Strategy for sequencing the 1,693-bp DNA fragment from the *Flavobacterium* plasmid pPDL2 containing the *opd* gene. Light arrows indicate DNA sequences from M13 subclones that were combined to yield the entire DNA sequence. Heavy arrow shows the ORF for the *opd* gene. Positions of the putative translation initiation site and actual amino terminus are marked Met and Ser, respectively. Restriction sites used to generate M13 subclones are shown (only one of many *Sau*3A sites is shown). The site of fusion between the *lacZ* and *opd* genes in plasmid pJK33 is marked ∇^1. The linker insertion site used to generate plasmid pJK33∇Dde is marked ∇^2. The length of the sequence in base pairs is shown.

with 7-deaza-dGTP and Sequenase used as described by the manufacturer.

Protein purification, amino-terminal sequencing, and amino acid composition analysis. Purification of the membrane-bound *Flavobacterium* hydrolase has been described elsewhere (10). The hydrolase proteins specified by pWM513 and pJK33 were purified from *E. coli* extracts, using essentially the procedure used for the *Flavobacterium* hydrolase except that the pWM513 and pJK33 hydrolases were isolated from the soluble fraction of cell extracts rather than from the membrane fraction. Before amino-terminal analysis and amino acid composition analysis, protein samples were further purified by reverse-phase chromatography, using a Brownlee RP300 column (Applied Biosystems) and a gradient of 0 to 67% acetonitrile–33% *n*-propanol in 0.1% trifluoroacetic acid. Amino-terminal residues of protein samples were determined by using 10-μg samples and an Applied Biosystems model 477A peptide sequencer. The amino acid composition of a 3-μg sample of the *Flavobacterium* hydrolase was determined by high-performance liquid chromatography separation of the amino acids from a 24-h hydrolysate in 6 N HCl at 108°C.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins was performed by the method of Laemmli (8), using 12% resolving gels and 4% stacking gels. The proteins in the gels were fixed and stained in a solution of 0.25% Coomassie brilliant blue R in 40% methanol–7% acetic acid. The gels were destained in 40% methanol–7% acetic acid.

Construction of the *lacZ-opd* fusion plasmid pJK33. To generate a series of *lacZ-opd* gene fusions, the 1.2-kb *Sph*I-*Pst*I fragment from pWM513 was digested with exonuclease S1 (3 mg/ml of DNA, 15,000 U/ml of enzyme, 37°C, 30 min). These digestion conditions allowed the digestion of both single- and double-stranded DNA (see Results). After extraction with phenol-chloroform and precipitation with ethanol, the DNA was subjected to blunt-end ligation with an excess of the *Eco*RI linker oligonucleotide 5′-GGAATTCC-3′ (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and then digested with *Bam*HI and *Eco*RI. After extraction with phenol-chloroform and precipitation with ethanol, the DNA was ligated to pUC19 DNA that had been digested with *Bam*HI and *Eco*RI and used to transform *E. coli* DH5α cells. Ampicillin-resistant transformants were screened for parathion hydrolase activity (10). DNA sequencing was used to determine the *lacZ-opd* fusion sites in the recombinant plasmids that were generated.

Mutagenesis of pJK33. pJK33 is an active *lacZ-opd* gene fusion plasmid (its construction is described above). The 910-base-pair (bp) *Eco*RI-*Dde*I DNA fragment from pJK33 was isolated and ligated to a *Dde*I-digested *Dde*I-*Hin*dIII linker oligonucleotide, 5′-TGAGGTAGAAGCTT-3′ (Synthecell Corp., Rockville, Md.). After ligation, the DNA was digested with *Hin*dIII, ligated to pUC19 DNA that had been digested with *Eco*RI and *Hin*dIII, and used to transform *E. coli* DH5α cells. Insertion of the oligonucleotide was verified by DNA sequencing the recombinant plasmid (pJK33∇Dde) that was generated.

RESULTS

Nucleotide sequence of the *Flavobacterium* plasmid fragment containing the *opd* gene. Figure 2 shows the nucleotide sequence of the 1,693-bp *Bam*HI-*Pst*I DNA fragment from the 39-kb *Flavobacterium* plasmid pPDL2. Previous studies have demonstrated the orientation and approximate location of the *opd* gene within the 1,168-bp region bounded by the *Pst*I site at position 357 and the *Bam*HI site at position 1525 (11, 12, 16). Within this region, there is only one open reading frame (ORF) large enough to encode the 35,000-dalton (Da) hydrolase protein (10). Upstream of this reading frame, a possible promoter was identified on the basis of its homology to the *E. coli* promoter consensus sequence at positions −35 and −10 (5). A possible Shine-Dalgarno sequence, AAGG, is located 7 bp upstream of the first ATG codon that occurs in the reading frame (19).

Amino-terminal analysis and amino acid composition of the *Flavobacterium* hydrolase. By using the first ATG codon (position 419) within the ORF as the presumptive translation initiation site of the *opd* gene, a protein of 39,000 Da is predicted from the nucleotide sequence. This value is considerably larger than the estimate of 35,000 Da obtained from chromatography and electrophoresis of the purified protein (10). One explanation for the discrepancy between the predicted and observed protein masses is that the hydrolase protein is translated as a larger polypeptide that is then subjected to posttranslational cleavage to yield a smaller, mature species. Indeed, the posttranslational processing of this membrane-bound protein may play an integral role in determining its ultimate cellular location. To test this hy-

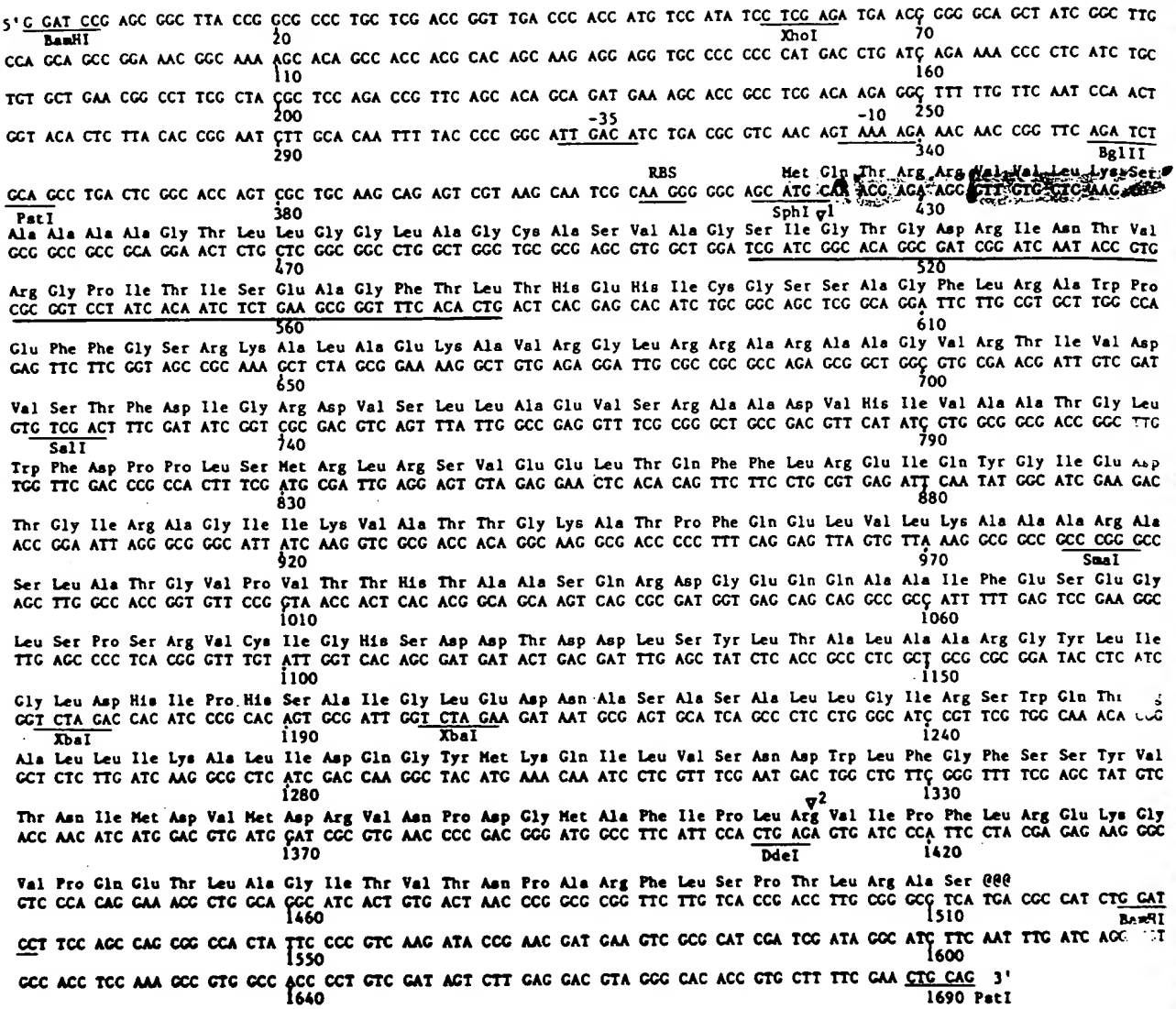

FIG. 2. Nucleotide and deduced amino acid sequences of the *Flavobacterium* parathion hydrolase gene *opd*. Putative promoter sequences (−35 and −10), a Shine-Dalgarno sequence (RBS), and sites of oligonucleotide insertion for constructing pJK33 (∇^1) and pJK33∇Dde (∇^2) are shown above the appropriate locations. The amino-terminal residues of the hydrolase protein that was purified from *Flavobacterium* extracts are underlined by a thick line. Restriction sites used in the DNA sequencing are underlined by thin lines.

pothesis, the hydrolase protein was purified from *Flavobacterium* extracts, and the first 24 amino-terminal residues were determined (Fig. 2). The sequence of the amino-terminal residues of the *Flavobacterium* hydrolase revealed that serine is the amino-terminal residue, corresponding to a TCG codon (position 506) 87 bp downstream of the presumptive ATG initiation codon in the DNA sequence (Fig. 2). Comparison of the amino acid composition of the purified protein with the composition predicted by the DNA sequence (using serine at position 506 as the amino terminus) showed close agreement between the two (Table 1). Thus, although we have no direct evidence of a precursor polypeptide in *Flavobacterium* extracts, the amino terminus of the mature protein indicates that it is the product of proteolytic cleavage. A plot of the hydropathic character of the polypeptide predicted from the DNA sequence revealed the hydrophobic nature of the putative leader peptide (Fig. 3).

Construction of *lacZ-opd* fusions. Even within cons[illegible] tions in which an exogenous promoter (such as the [illegible]Z promoter in pWM513) was inserted upstream of *opd*, parathion hydrolase activity was markedly lower in *E. coli* extracts than in extracts from wild-type *Flavobacterium* cells (Table 2). It is possible that the parathion hydrolase encoded by *opd* is incorrectly processed from its larger precursor in *E. coli* and that proper processing is a prerequisite for optimal enzyme activity. This proposal is supported by comparison of the hydrolase protein isolated from *E. coli* cells containing pWM513 (in which the 1.3-kb *Pst*I fragment containing the *opd* gene is inserted into pU[illegible]) with the hydrolase protein from *Flavobacterium* extracts. First, unlike membrane-bound enzyme in *Flavobacterium* sp., the pWM513 enzyme from *E. coli* extracts was primarily located in the cytosol (Table 2). Second, the pWM513 enzyme was larger than that isolated from *Flavobacterium*

TABLE 1. Comparison of the experimental and predicted amino acid compositions of the *Flavobacterium* sp. hydrolase protein

Amino acid	Molar composition (%)		Predicted no. of residues[a]
	Experimental	Predicted[a]	
Asn + Asp	7.61	7.44	6 + 19 = 25
Gln + Glu	8.39	7.74	10 + 16 = 26
Ser	7.65	7.74	26
Gly	8.66	8.33	28
His	2.01	2.08	7
Arg	7.81	7.74	26
Thr	7.63	7.44	25
Ala	12.60	11.61	39
Pro	4.65	4.17	14
Tyr	1.57	1.49	5
Val	6.11	6.55	22
Met	1.65	1.49	5
Cys	0.44[b]	0.60	2
Ile	5.86	7.74	26
Leu	10.17	9.82	33
Phe	4.51	4.46	15
Lys	2.68	2.38	8
Trp	—[c]	1.20	4

[a] Predicted from the DNA sequence, using serine as the amino-terminal residue.
[b] Recovery of Cys by this method is typically quite low relative to levels of other residues and therefore is of little comparative value.
[c] Trp is destroyed during hydrolysis and therefore cannot be quantitated.

TABLE 2. Comparison of parathion hydrolase activities of extracts of *Flavobacterium* sp. and *E. coli* containing *opd* plasmids[a]

Host strain	Plasmid	Sp act (μmol·min^{-1}·mg of protein^{-1})[b]	% of total activity
Flavobacterium sp.	pPDL2		
Crude extract		6.35	
Soluble fraction		3.10	36
Membrane fraction		17.35	64
E. coli DH5α	pWM513		
Crude extract		0.10	
Soluble fraction		0.11	82
Membrane fraction		0.04	18
E. coli DH5α	pJK33		
Crude extract		0.28	
Soluble fraction		0.33	91
Membrane fraction		0.11	9
E. coli DH5α, crude extract	pJK33∇Dde	ND	

[a] Cell extracts from 500-ml cultures of *Flavobacterium* sp. and *E. coli* were prepared as previously described (10).
[b] Detection limit of the assay is <0.00002 μmol · min^{-1} · mg of protein^{-1}. ND, No detectable activity.

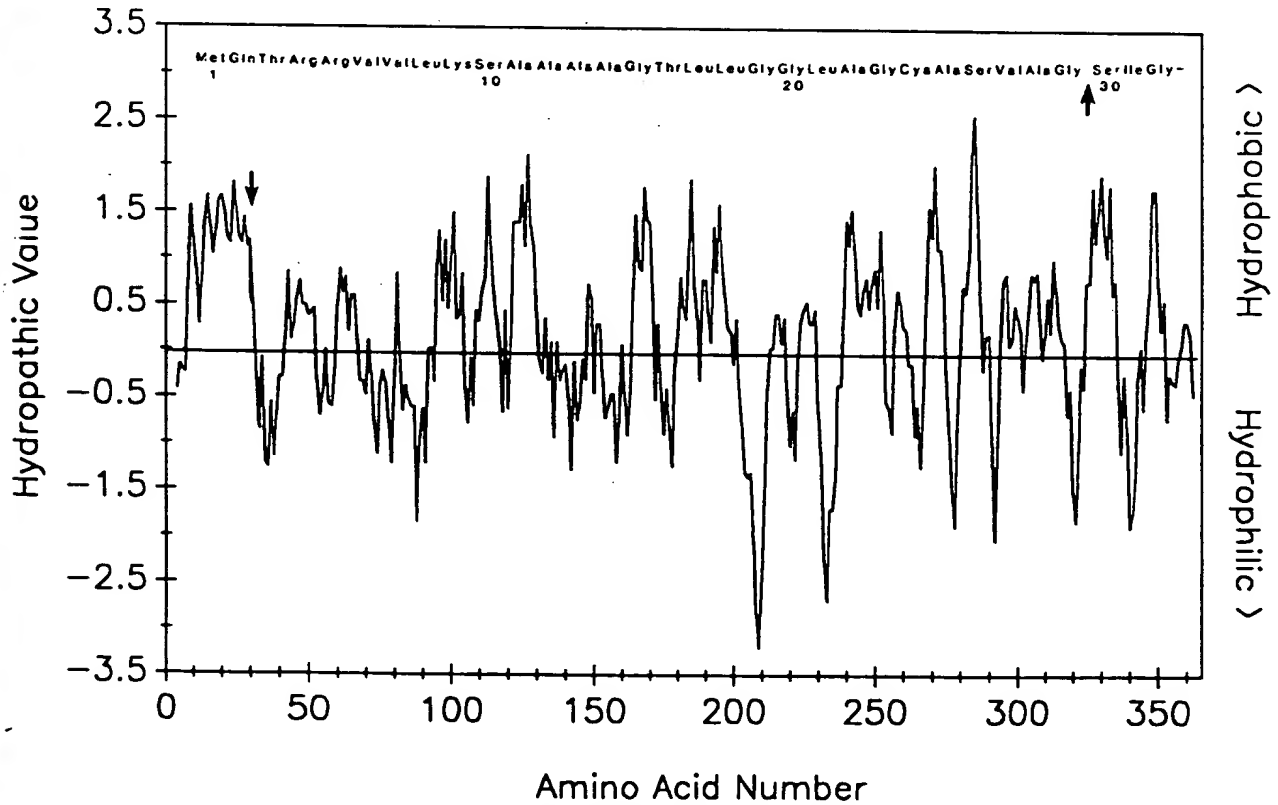

FIG. 3. Plot of the hydrophobic character of the proposed parathion hydrolase precursor polypeptide. Values were calculated for amino acids deduced from the DNA sequence, using an averaging length of seven residues (7). The deduced amino acid sequence of the first 32 residues of the precursor polypeptide is shown above the plot. The leader peptide cleavage site is marked by a vertical arrow.

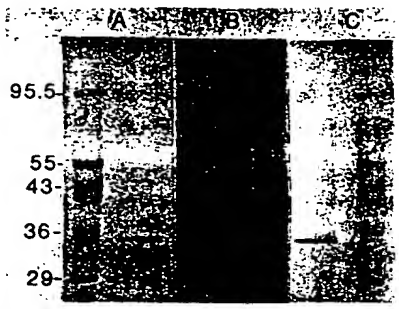

FIG. 4. Comparison of the parathion hydrolase protein from *Flavobacterium* sp. strain ATCC 27551 containing pPDL2 with the hydrolase proteins produced in *E. coli* strains containing pWM513 and pJK33. Purified proteins from each strain were subjected to SDS-PAGE as described in the text. Molecular mass values of protein standards are expressed in kilodaltons. (A) *Flavobacterium* sp.(pPDL2) hydrolase; B, *E. coli*(pWM513) hydrolase; (C) *E. coli* (pJK33) hydrolase.

sp. (Fig. 4). Although it is likely that the pWM513 enzyme corresponds to the predicted hydrolase precursor protein, we have been unable to directly test this hypothesis by sequencing the amino-terminal residues of the pWM513 protein.

If improper (or lack of) processing of the hydrolase in *E. coli* is the basis of the low activity in cells containing pWM513, then deletion of the coding region for the signal peptide could lead to increased parathion hydrolase activity in *E. coli* extracts. To test this hypothesis, *lacZ-opd* fusions were constructed in which the 5' end of the *opd* gene was partially digested and fused to the first five codons of *lacZ* in pUC19. In one of the resulting hybrid plasmids (pJK33), the first five codons of *lacZ* were fused to the *opd* codon GGC (Gly) at position 518 (Fig. 2), thus deleting the 29-residue signal peptide along with the first four residues of the native mature protein. Amino-terminal sequencing of the hydrolase protein from extracts of cells containing pJK33 confirmed that this fusion protein contained the first five *lacZ* residues (Met-Ile-Thr-Asn-Ser-) followed by hydrolase residues (-Gly-Asp-Arg-). Extracts of *E. coli* cells containing pJK33 showed levels of hydrolase twofold higher than those of cells containing the parent plasmid pWM513 (Table 2). Moreover, the pJK33 *lacZ-opd* fusion protein was identical in mass to the *Flavobacterium* protein, as judged by SDS-PAGE (Fig. 4). That the two proteins had the same mass suggests that the *Flavobacterium* hydrolase is not processed further after cleavage of the leader peptide.

Mutagenesis of the carboxyl region of the *Flavobacterium* hydrolase. The results of our nucleotide-sequencing experiments on the *Flavobacterium opd* gene showed 50 differences from the results of a previous study (Fig. 5) (4). One result of the many differences between the two sequences is that they predict different translation termination points for the hydrolase protein. To test whether the translation of the *Flavobacterium* hydrolase terminates before the point predicted by our nucleotide sequence, a mutant of the *lacZ-opd* fusion gene in pJK33 was constructed by inserting a TAG nonsense codon 3' to the *opd Dde*I site at position 1406 (Fig. 2). Consistent with the predictions of our sequence, *E. coli* cells containing the mutant plasmid (pJK33-∇Dde) displayed no hydrolase activity (Table 2).

DISCUSSION

This study presents the DNA sequence of the *opd* gene from the 39-kb *Flavobacterium* plasmid pPDL2. Although the sequence we determined varies substantially from a previously reported sequence (4), our sequence is fully consistent with the experimentally determined amino-terminal sequence and amino acid composition of the purified protein. We give evidence that the *Flavobacterium* enzyme is processed at its amino terminus. In addition, we show that deletion of the nucleotide sequence encoding the putative leader peptide increases activity of the gene in *E. coli* extracts.

Examination of the *opd* gene DNA sequence reveals one ORF capable of encoding the 35,000-Da hydrolase protein. A search of the DNA sequence upstream of the putative *opd* coding region revealed putative promoter and Shine-Dalgarno sequences with appropriate spacing. In fact, the sequences found are a perfect match for the −35 consensus sequence (GGTACA) and a less favorable match at −10 (TAAAAG). If these sequences are used in *Flavobacterium* sp. as an RNA polymerase promoter, then it is unclear why they do not function in *E. coli*. Nevertheless, expression of the *opd* gene in *E. coli* is entirely dependent on exogenous promoters (6; W. Mulbry, Ph.D. dissertation, University of Maryland, College Park, 1987). It is possible that there is a transcription termination sequence downstream of this pro-

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455      463....493   497...523   527...533          544...585          596...642  645...657  660...681      689...788   792
  GCCGCAGGA         GAGCG        TCGGA       ACCGTGCGCGGT        AGCACATCTGCG       GCCG       CGGA       GCCGCCGCCA       ATCGT
   * * *              *            **           *    *              *    *             *          *           *  **           *
  GC G A GA         GA CG        TCCGA       AT GTGCGCG T        AGGACATCT CG       GC G       CGCA       GC GC  CA       ATC
99       104....134   137...163   167...173          182...223          233...279  281...293  296...317      322...421

840   844...859   862...872   876...895   898...1048      1053...1075   1079...1145          1158...1225   1230...1402  1405
  CGAGT        AC AG        CG TGA        AG AC        GC AG CA          AGGCT        TCGCTGCGCGCGG          AGCCCT         TCCA
   **           *            *             *             * *              *             *  **              ***           *
  CGTAT        ACTAG        CGGTGA        AGTAC        GCGAGGCA          AG CT        T GCTG  CGCGG          ACCGCT         T CA
472   476...491   495...505   510...529   533....683      690.....712   715.....781          790.....857   862....1034  1036

1422  1425...1489   1492..1500          1512...1535   1539...1605  1608..1633  1635...1659                              1687
  TCCT         CT TG        CCTTGCGGGCGT C          AGCCA         ATTT        CG T        AGG ACG TAGGCCACACCGTGCTTTTC  CAA
   *            *            *   ***  *            **            *           *            *  *  *  * ***              **
  TC T         CTGTG        C TTGC   CGTGC          ACGCA         ATGC        CGGT        AGGCACGGTAGCGACGACCGTGCTTTTGTG
1053  1055...1119   1123..1131          1140...1163   1167...1233  1236..1261  1264...1288                              1320
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FIG. 5. Differences between the *Flavobacterium* nucleotide sequence determined in this study and that reported by Wild and co-workers (4). The upper nucleotide sequence and numbering system correspond to the sequence determined in this study; the lower nucleotide sequence and numbering system correspond to the sequence reported by Wild and co-workers (4). Positions where the two sequences differ are marked (*).

moter sequence that is recognized in *E. coli* but not in *Flavobacterium* sp. However, this appears unlikely, since insertion of an exogenous promoter upstream of these sequences (at the *Bam*HI site [position 1]; 16) activates the *opd* gene at levels roughly equivalent to those of constructions in which the promoter is inserted downstream of these sequences (at the *Pst*I site [position 357]). Recently, the 1.5-kb *Bam*HI fragment that contains the *Flavobacterium opd* gene has been shown to function in the gram-positive organism *Streptomyces lividans* in the absence of exogenous promoter sequences (18). The responsible promoter sequences were localized by a deletion experiment to the region that contains our putative promoter sequences.

Wild and co-workers (4) have noted the presence of a sequence 5' to the *opd* coding region that bears some resemblance to a *nif*-type promoter sequence (3). This sequence is also present in the nucleotide sequence we have determined. We have cloned a 7.3-kb *Eco*RI fragment that encompasses nearly the entire region conserved between the *opd*-containing plasmids (12) in the broad-host-range vector pVDZ'2 (2). When this construct was introduced into *Pseudomonas putida*, the cells showed little or no parathion hydrolase expression (J. S. Karns, unpublished observations). Thus, if the promoter postulated by Wild and co-workers is responsible for initiating transcription of the *opd* gene in *Flavobacterium* sp., it does not function in *P. putida*. The activity of such promoters is frequently positively regulated, and the lack of a positive regulatory element may explain the lack of parathion hydrolase expression in gram-negative hosts other than the native *Flavobacterium* sp. and *P. diminuta*. However, since the conserved region between the two plasmids was cloned, it seems that any positive regulator unique to *opd* expression would also have been cloned. The lack of expression in *P. putida* combined with the apparently constitutive expression of *opd* in *Flavobacterium* sp. and *P. diminuta* argue against any such regulated promoter.

The putative Shine-Dalgarno sequence AAGG is located 7 bp upstream of the first ATG codon that occurs in the *opd* ORF. However, amino-terminal sequencing of the hydrolase protein shows that the amino-terminal residue is a serine located 29 residues downstream of this Met codon. The putative 29-residue leader peptide, like other leader peptides (20), is hydrophobic and has basic residues near its amino terminus. However, the cleavage site used by *Flavobacterium* cells would not be predicted by current models (there would be several other preferred sites) (20). The isolation from *E. coli* extracts of a hydrolase protein that is larger than that from *Flavobacterium* extracts suggests that the signal peptide is not cleaved in *E. coli*. In contrast, the signal peptide may be functional in gram-positive organisms—when the 1.5-kb *Bam*HI fragment containing the *Flavobacterium opd* gene is cloned in *S. lividans*, parathion hydrolase is secreted into the culture medium (18).

The predicted mass of the processed *opd* hydrolase (36,000 Da) differs slightly from the observed mass of the protein from *Flavobacterium* extracts (35,000 Da). Despite this difference, the veracity of the nucleotide sequence is supported by agreement of the predicted and experimental amino acid compositions, confirmation of amino-terminal sequence, and results of a deletion cloning experiment (in which expression was eliminated by the insertion of an in-frame nonsense codon about 100 bp upstream of the predicted carboxyl terminus). Therefore, it is likely that the discrepancy between predicted and observed mass is probably due to the aberrant mobility of the hydrolase during electrophoresis and gel filtration chromatography.

Relatively low expression of the *Flavobacterium opd* gene even in the presence of exogenous *E. coli* promoters lead to an examination of the *opd* protein produced in *E. coli*. The *E. coli* protein is larger than the protein isolated from *Flavobacterium* extracts and is therefore either an unprocessed or a misprocessed form of the precursor protein. Moreover, in contrast to the membrane-associated *Flavobacterium* protein, the *E. coli* protein is primarly partitioned in the cytosol. Extracts of *E. coli* cells containing a *lacZ-opd* translational fusion in which the leader peptide was deleted and replaced by five amino-terminal residues of *lacZ* displayed greater hydrolase activity than did cells that contained the entire *opd* gene.

Wild and co-workers previously reported the sequence of the 1.3-kb *Pst*I fragment from the *Flavobacterium* plasmid pPDL2 (erroneously termed pSM55 in their work) (4). Although their sequence (corresponding to positions 357 to 1693 in Fig. 2) agrees in large part with the sequence we determined, there are 50 base differences between the two in the region that they overlap. The reason for these substantial differences (which lead to significantly different predicted hydrolase proteins) is unclear. It is unlikely that these changes stemmed from cloning from different original sources, since M13 subclones used for both studies were derived from pWM44. The predictions of our nucleotide sequence agree well with experimentally determined values for amino-terminal sequence, amino acid composition, and approximate site of carboxyl terminus. In contrast, the predictions from the nucleotide sequence of Wild and co-workers are not consistent with any of these experimental values.

Southern hybridization experiments as well as restriction mapping of the *Flavobacterium opd* plasmid pPDL2 and *P. diminuta opd* plasmid pCMS1 have shown that the two *opd* genes are related if not identical (11, 12). More recently, Wild and co-workers reported the nucleotide sequence of the *Pseudomonas opd* gene (9). This sequence was identical to the sequence they later determined for the *Flavobacterium* gene with the exception of a single-base change (4, 9). Although we have no independent sequence information for the *Pseudomonas opd* gene or any protein work to test their sequence, the two genes are probably identical. Therefore, it appears likely that the *Pseudomonas opd* nucleotide sequence of Wild and co-workers is in error in precisely the same regions as is their *Flavobacterium opd* nucleotide sequence. A more thorough analysis of the *Pseudomonas opd* nucleotide sequence will undoubtably resolve this question.

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ADDENDUM IN PROOF

Through personal communication we have learned that Cüneyt Serdar and his colleagues at AMGEN have determined the correct nucleotide sequence of the *Pseudomonas diminuta opd* gene. Their work (Bio/Technology, in press)

shows that within the protein-coding region, the nucleotide sequence of the *P. diminuta opd* gene is identical to the *Flavobacterium* sequence we have presented here.

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CHAPTER 11

Recombination at the Molecular Level

All DNA is recombinant DNA. In engineering relatively simple changes in gene expression by combining and modifying DNA segments in the test tube, we are only simulating natural processes of recombination and mutation that have acted throughout evolution to construct complex chromosomes. Genetic exchange works constantly to blend and rearrange chromosomes, most obviously during meiosis, when homologous chromosomes cross over in every generation. Genetic maps derived from early measurements of the frequency of crossing over between different genes gave the first real information about chromosome structure: that genes are arranged in a fixed, linear order. A most important recent discovery is that gene order does change, although rarely: Movable DNA segments called transposons occasionally jump around chromosomes, thus fundamentally altering chromosomal structure. In addition to neatly moving genes, transposons also scramble DNA, making deletions, inversions, and other rearrangements. It is becoming clear that such changes are a critical feature of chromosome evolution, particularly in eucaryotic cells.

We now appreciate that recombination is not accidental, but is instead an essential cellular process catalyzed by enzymes that cells encode and regulate for the purpose. Besides providing genetic variation, recombination enzymes allow cells to retrieve sequences lost when DNA is damaged by radiation or chemical accidents, by replacing the damaged section with an undamaged DNA strand from a homologous chromosome. Furthermore, special types of recombination regulate gene expression. By switching specific segments within chromosomes, cells put dormant genes into sites where they can be expressed, even creating new protein-coding regions. Moreover, recent discoveries, in revealing the molecular mechanisms of recombination, have provided new tools for the deliberate manipulation of genes.

Crossing Over Is Due to Breakage and Rejoining of Intact DNA Molecules[1]

Until recently, there was not even a superficial understanding of the molecular basis of crossing over. The classical picture of crossing over, developed in the 1930s from cytological observations, hypothesized that during meiosis, the paired, coiled chromosomes were sometimes physically broken at the chromatid level as a result of ten-

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Chapter 5

Patterns of Folding and Association of Polypeptide Chains

Six levels of structural organization can be distinguished. According to Linderström-Lang (176), four levels of structural organization in proteins can be distinguished: primary, secondary, tertiary, and quaternary structure (176). These terms refer to the amino acid sequence, the regular arrangements of the polypeptide backbone, the three-dimensional structure of the globular protein, and the structures of aggregates of globular proteins, respectively. With our present knowledge, two more levels can be added: supersecondary structures denoting physically preferred aggregates of secondary structure and domains referring to those parts of the protein which form well-separated globular regions. An organizational scheme is given in Figure 5-1a. Since renaturation experiments have shown that the amino acid sequence contains the entire structural information (177), the relationship between these levels is dependent, with elements at a lower level determining the elements of higher levels.

This relationship has been schematized in an extreme simplification in Figure 5-1b. This scheme assumes that all interactions are well-segregated; there is no interference from elements nonadjacent along the polypeptide chain, or from elements of other levels. The scheme resembles a hierarchic social system (with reversed polarity). In the following we will describe all levels and finally discuss the relationships between them.

5.1 Secondary Structure

Secondary structures are regular arrangements of the backbone of the polypeptide chain without reference to the side chain types or conformations. They are stabilized by hydrogen bonds between peptide amide and carbonyl groups.

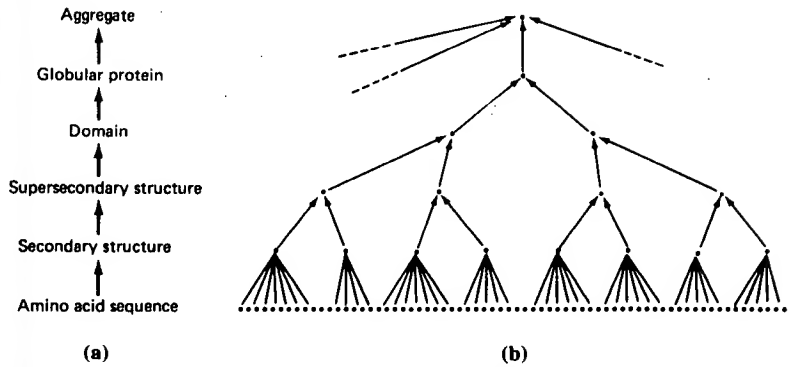

Figure 5-1. Levels of structural organization in globular proteins.

Linear Groups

The backbone of a polypeptide chain forms a linear group if its dihedral angles are repeated. Every linear group is a helix. If the regularity is such that successive peptide units assume identical relative orientations, that is, if all (ϕ,ψ)-angles (Figure 2-2) are the same, the polypeptide backbone forms a linear group. Every linear group is a helix. Helices are conveniently described by the rise per element d, the number of elements per turn n, and the distance r of a marker point on each element (here the C_α-atom) from the helix axis (Figure 5-2). Since d is taken as positive, the helix chirality can be read from the sign of n. Each helix has a polarity, because the peptide unit is polar.

The relationship between (ϕ,ψ)-angles (Figure 2-2) and helix parameters d and n is given in Figure 5-3. No helix with $|\, n \,| < 2$ is possible. There are only a few linear groups without steric hindrance that are stabilized by hydrogen bonds, either within a chain (e.g., α-helix) or between neighbor-

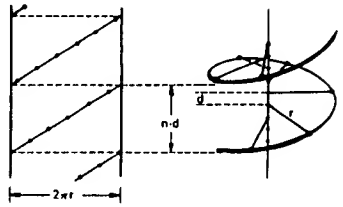

Figure 5-2. Helix with helix parameters and surface net representation: n = residues per turn, d = axial shift per residue, $n \cdot d$ = pitch of the helix, r = radius of the helix. The surface net is produced by projection of the helix onto a coaxial cylindrical sheet of paper, cutting this paper parallel to the helix axis and flattening it. The surface net is also called cylindrical plot. It allows visualization of the geometrical relationship between residues. Reversing the viewing direction onto the cut paper leads to the mirror image of the given cylindrical plot which is also in use.

II

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dis·so·cia·ble (di-sō'shə-bəl, -shē-ə-bəl) *adj.* Capable of being dissociated : SEPARABLE. **—dis·so'cia·bil'i·ty, dis·so'cia·ble·ness** *n.* **—dis·so'cia·bly** *adv.*

dis·so·ci·ate (di-sō'shē-āt', -sē-) *v.* **-at·ed, -at·ing, -ates.** [Lat. *dissociare, dissociat-* : *dis-* (reversal) + *sociare,* to unite < *socius,* companion.] —*vt.* **1.** To remove from association : SEPARATE <"Marx never *dissociated* man from his social environment" —Sidney Hook> **2.** *Chem.* To cause to undergo dissociation. —*vi.* **1.** To cease associating : PART. **2.** *Chem.* To undergo dissociation. **—dis·so'ci·a'tive** *adj.*

dis·so·ci·a·tion (di-sō'sē-ā'shən, -shē-) *n.* **1.** The act of dissociating or state of being dissociated. **2.** *Chem.* **a.** The process by which a change in physical condition, as in temperature or pressure, or the action of a solvent causes a molecule to split into less complex groups of atoms, single atoms, or ions. **b.** The separation of an electrolyte into ions of opposite sign. **3.** *Psychiat.* The separation of a group of related psychological activities into autonomously functioning units, as in the generation of multiple personalities.

dis·sol·u·ble (di-sŏl'yə-bəl) *adj.* [Lat. *dissolubilis* < *dissolvere,* to dissolve.] Capable of being dissolved. **—dis'sol·u·bil'i·ty, dis·sol'u·ble·ness** *n.*

dis·so·lute (dis'ə-lōōt') *adj.* [ME < Lat. *dissolutus,* p.part. of *dissolvere,* to dissolve.] Lacking moral restraint : PROFLIGATE. **—dis'so·lute'ly** *adv.* **—dis'so·lute'ness** *n.*

dis·so·lu·tion (dis'ə-lōō'shən) *n.* **1.** Disintegration into component parts : DECOMPOSITION. **2.** Lack of moral restraint. **3.** Termination or extinction by deconcentration or dispersion. **4.** Death. **5.** Annulment or termination of a formal or legal bond, tie, or contract. **6.** Formal adjournment or dismissal of an assembly or legislature. **7.** Reduction to a liquid form. **—dis'so·lu'tive** *adj.*

dis·solve (di-zŏlv') *v.* **-solved, -solv·ing, -solves.** [ME *dissolven* < Lat. *dissolvere* : *dis-,* apart + *solvere,* to release.] —*vt.* **1.** To cause to pass into solution <*dissolve* instant coffee in water> **2.** To reduce to liquid form : MELT. **3.** To cause to disappear : DISPEL. **4.** To separate into component parts : DISINTEGRATE. **5.** To bring to an end by or as if by breaking up : TERMINATE. **6.** To dismiss (e.g., an assembly or legislature). **7.** To affect emotionally. **8.** To cause to lose definition : BLUR <"Morality has finally been *dissolved* in pity" —Leslie Fiedler> **9.** *Law.* To render null : ABROGATE. —*vi.* **1.** To pass into solution. **2.** To melt. **3.** To disperse or break up. **4.** To become disintegrated. **5.** To be moved emotionally <*dissolved* in tears> **6.** To lose definition or clarity : fade away. **7.** To shift scenes in a motion-picture film or videotape by having one scene fade out while the next appears behind it and grows clearer as the first dims. —*n.* A scene transition in a motion-picture film or videotape made by dissolving. **—dis·solv'a·ble** *adj.* **—dis·solv'er** *n.*

dis·sol·vent (di-zŏl'vənt) *n.* A solvent. **—dis·sol'vent** *adj.*

dis·so·nance (dis'ə-nəns) *also* **dis·so·nan·cy** (-nən-sē) *n.* **1.** A harsh or unpleasant combination of sounds : DISCORD. **2.** Lack of agreement : CONFLICT. **3.** *Mus.* A combination of tones conventionally held to suggest unrelieved tension and to require resolution.

dis·so·nant (dis'ə-nənt) *adj.* [ME *dissonaunt* < OFr. *dissonant* < Lat. *dissonans,* pr.part. of *dissonare,* to be dissonant : *dis-,* apart + *sonare,* to sound.] **1.** Harsh or unpleasant in sound : DISCORDANT. **2.** Disagreeing : conflicting. **3.** *Mus.* Constituting or producing a dissonance. **—dis'so·nant·ly** *adv.*

dis·suade (di-swād') *vt.* **-suad·ed, -suad·ing, -suades.** [Lat. *dissuadēre* : *dis-* (reversal) + *suadēre,* to advise.] To discourage or deter from a course of action or intention by exhortation or persuasion. **—dis·suad'er** *n.*

dis·sua·sion (di-swā'zhən) *n.* [Lat. *dissuasio* < *dissuadēre,* to dissuade.] The act or an instance of dissuading. **—dis·sua'sive** *adj.* **—dis·sua'sive·ly** *adv.* **—dis·sua'sive·ness** *n.*

dis·syl·la·ble (di-sĭl'ə-bəl, dis'sĭl-, dĭ'sĭl-) *n. var. of* DISTLLABLE.

dis·sym·me·try (dis-sĭm'ĭ-trē) *n., pl.* **-tries.** Lack of symmetry. **—dis'sym·met'ric** (dĭ'sĭ-mĕt'rĭk), **dis'sym·met'ri·cal** *adj.* **—dis'sym·met'ri·cal·ly** *adv.*

dis·taff (dis'tăf') *n.* [ME *distaf* < OE *distæf* : *dis-,* bunch of flax + *stæf,* staff.] **1.** A staff having a cleft end that holds the unspun flax, wool, or tow from which thread is drawn in spinning by hand. **2.** A woman's work and domain. **3.** Women as a group.

distaff side *n.* The maternal branch or female side of a family.

dis·tal (dis'təl) *adj.* [DIST(ANT) + -AL.] *Anat.* Located far from the origin or line of attachment, as a bone. **—dis'tal·ly** *adv.*

dis·tance (dis'təns) *n.* **1.** The fact or condition of being apart in space or time. **2. a.** A nonnegative number designating the magnitude of a path along a straight line or curve. **b.** The length of a line segment joining two points. **c.** The length of the perpendicular from a given point to a given line. **3.** The interval separating two specified instants in time. **4.** The extent of space between points on a linearly measured course. **5. a.** The degree of deviation or difference that separates two things in relationship <the *distance* between liberal and conservative> **b.** The degree of progress between two points in a course or trend. **6.** A stretch of linear space without definite limits. **7.** A point removed in space or time. **8.** Aloofness of manner : RESERVE. —*vt.* **-tanced, -tanc·ing, -tanc·es. 1.** To place or keep at a distance. **2.** To cause to appear at a distance. **3.** To leave far behind : OUTSTRIP.

dis·tant (dis'tənt) *adj.* [ME *distaunt* < OFr. < Lat. *distans,* pr.part. of *distare,* to be remote : *dis-,* apart + *stare,* to stand.] **1.** Apart or separate in space or time. **2.** Far removed in space or time <the *distant* future> **3.** Located at, coming from, or going to a distance <*distant* travels> **4.** Remotely related <a *distant* cousin> **5.** Of or relating to mental distance or absent-mindedness <a *distant* reverie> **6.** Reserved in manner : ALOOF. **—dis'tant·ly** *adv.*

dis·taste (dis-tāst') *n.* Dislike or aversion. —*vt.* **-tast·ed, -tast·ing, -tastes.** *Archaic.* **1.** To feel repugnance for. **2.** To offend.

dis·taste·ful (dis-tāst'fəl) *adj.* **1. a.** Disagreeable or unpleasant <the *distasteful* job of laying off workers> **b.** Objectionable or offensive <*distasteful* magazines> **2.** Expressing distaste <a *distasteful* glare> **—dis·taste'ful·ly** *adv.* **—dis·taste'ful·ness** *n.*

dis·tem·per[1] (dis-tĕm'pər) *n.* [ME *distemperen,* to upset the balance of the humors < OFr. *destemprer* < Med. Lat. *distemperare* : Lat. *dis-* (reversal) + Lat. *temperare,* to temper.] **1. a.** An infectious virus disease occurring in certain mammals, esp. dogs, marked by loss of appetite, a catarrhal discharge from the eyes and nose, and often partial paralysis and death. **b.** Any of various similar mammalian diseases. **2.** Bad temper : PEEVISHNESS. **3.** Social or political disorder. —*vt.* **-pered, -per·ing, -pers.** To upset.

dis·tem·per[2] (dis-tĕm'pər) *n.* [ME *distemperen,* to dilute < Med. Lat. *distemperare.* —see DISTEMPER[1].] **1. a.** A process of painting in which pigments are mixed with water and a glue-size or casein binder, used for flat wall decoration or for scenic and poster painting. **b.** The paint used in distemper. **2.** A painting done in distemper. —*vt.* **-pered, -per·ing, -pers. 1.** To mix (powdered pigments or colors) with water and size. **2.** To paint in distemper.

dis·tend (di-stĕnd') *v.* **-tend·ed, -tend·ing, -tends.** [ME *distenden* < Lat. *distendere* : *dis-,* apart + *tendere,* to stretch.] —*vi.* To swell out or expand from or as if from internal pressure. —*vt.* **1.** To cause to expand by or as if by internal pressure : DILATE. **2.** To stretch out in all directions : EXTEND.

dis·ten·si·ble (di-stĕn'sə-bəl) *adj.* Capable of being distended. **—dis·ten'si·bil'i·ty** *n.*

dis·ten·tion *also* **dis·ten·sion** (di-stĕn'shən) *n.* [ME *distencioun* < Lat. *distentio* < *distentus,* p.part. of *distendere,* to distend.] The act of distending or state of being distended.

dis·tich (dis'tik) *n., pl.* **-tichs.** [Lat. *distichon* < Gk. *distikhon* < *distikhos,* having two rows or verses : *di-,* two + *stikhos,* line of verse.] A verse couplet, esp. one used in a Latin or Greek elegiac.

dis·ti·chous (dĭs'tĭ-kəs) *adj.* [LLat. *distichus,* having two rows < Gk. *distikhos.* —see DISTICH.] Arranged in two vertical rows or ranks on opposite sides of an axis. —Used of leaves. **—dis'ti·chous·ly** *adv.*

dis·til (dĭ-stĭl') *v. Chiefly Brit. var. of* DISTILL.

dis·till (dĭ-stĭl') *v.* **-tilled, -till·ing, -tills.** [ME *distillen* < OFr. *distiller* < Lat. *destillare,* to trickle : *de-,* down + *stillare,* to drip < *stilla,* drop.] —*vt.* **1.** To subject (a substance) to distillation. **2.** To extract (a distillate) by distillation. **3.** To refine or purify by or as if by distillation. **4.** To separate or extract the essence of <*distill* the main ideas of a film> **5.** To exude or give off in drops. —*vi.* **1.** To undergo or be produced by distillation. **2.** To fall or exude in drops. **—dis·till'a·ble** *adj.*

dis·til·late (dĭs'tə-lāt', -lĭt, dĭ-stĭl'ĭt) *n.* **1.** The liquid condensed from vapor in distillation. **2.** An essence or purified form.

dis·til·la·tion (dĭs'tə-lā'shən) *n.* **1.** Any of various heat-dependent processes used to purify or separate a fraction of a relatively complex mixture or substance, esp. the vaporization of a liquid mixture with subsequent collection of components by differential cooling to condensation. **2.** A distillate.

distillation column *n.* A tall cylindrical metal shell internally fitted with perforated horizontal plates used to promote separation of miscible liquids ascending in the shell as vapor.

dis·till·er (dĭ-stĭl'ər) *n.* **1.** One that distills, as a condenser. **2.** A maker of alcoholic liquors by distillation.

dis·till·er·y (dĭ-stĭl'ə-rē) *n., pl.* **-ies.** A plant or establishment for distilling, esp. alcoholic liquors.

dis·tinct (dĭ-stĭngkt') *adj.* [ME < OFr. < Lat. *distinctus,* p.part. of *distinguere,* to distinguish.] **1.** Distinguished from all others : INDIVIDUAL <met us on three *distinct* days> ***usage:*** Something is *distinct* if it is sharply distinguished or set apart from other things; a characteristic or property is *distinctive* if it enables us to distinguish one thing from another. **2.** Easily perceived : CLEAR <a *distinct* fragrance> **3.** Unquestionable : decided <a *distinct* drawback> **4.** Very likely : PROBABLE <a *distinct* chance of rain> **5.** Marked by excellence : NOTABLE <a *distinct* achievement> **—dis·tinct'ly** *adv.* **—dis·tinct'ness** *n.*

dis·tinc·tion (dĭ-stĭngk'shən) *n.* **1.** The act of distinguishing : DIFFERENTIATION. **2.** The condition or fact of being dissimilar : DIFFERENCE. **3.** A distinguishing factor, attribute, or characteristic. **4. a.** Excellence or eminence, as of performance, character, or reputation. **b.** A special feature or quality conferring superiority : VIRTUE. **5.** Recognition of achievement or superiority : HONOR <served their country with *distinction*>

mite² (mīt) *n.* [ME < MDu., a small Flemish coin.] **1. a.** A very small amount of money or contribution. **b.** A widow's mite. **2.** A coin of little value, esp. an obsolete British coin worth half a farthing. **3.** A very small object or creature.

mi·ter (mī'tər) *n.* [ME *mitre* < OFr. < Lat. *mitra*, headband < Gk.] **1.** A tall pointed hat with peaks in front and back, worn by bishops and certain other ecclesiastics. **2. a.** A thong for binding the hair, worn by ancient Greek women. **b.** The ceremonial headdress of ancient Jewish high priests. **3.** A covering or top of a chimney allowing the release of smoke while keeping out rain and debris. **4. a.** A miter joint. **b.** The edge of a piece of material that has been beveled preparatory to making a miter joint. **c.** A miter square. —*v.* **-tered, -ter·ing, -ters.** —*vt.* **1.** To bestow a miter upon. **2.** To make join with a miter joint. —*vi.* To meet in a miter joint.

miter box *n.* **1.** A box open at the ends, with slotted sides that guide a saw in cutting miter joints. **2.** A device for handsaws that may be set to guide cuts in lumber at various degrees.

miter joint *n.* A joint made by beveling each of two surfaces to be joined, usu. at a 45° angle, to form a 90° corner.

miter square *n.* A carpenter's square with a blade that is set at a 45° angle or is adjustable.

mi·ter·wort (mī'tər-wûrt', -wôrt') *n.* A North American plant of the genus *Mitella*, with heart-shaped leaves and small white flower clusters.

Mith·ra·ism (mith'rə-iz'əm, -rā-) *n.* A Persian religious cult that flourished in the late Roman Empire, rivaling Christianity. **—Mith·ra'ic** (-rā'ik) *adj.* **—Mith·ra'ist** (-rā'ist) *n.*

Mith·ras (mith'rəs) *n.* [Lat. < Gk. < OPers. *mithra.*] *Myth.* The Persian god of light and guardian against evil.

mith·ri·date (mith'ri-dāt') *n.* [After *Mithridates* (132?–63 B.C.), who is said to have acquired tolerance for poison.] A substance regarded as an antidote against poison.

mith·ri·da·tism (mith'ri-dā'tiz'əm) *n.* Tolerance for a poison acquired by taking ever larger doses. **—mith'ri·dat'ic** (-dăt'ik) *adj.*

mit·i·gate (mit'i-gāt') *vt.* & *vi.* **-gat·ed, -gat·ing, -gates.** [ME *mitigaten* < Lat. *mitigare* < *mitis*, soft.] To make or become less severe or intense : MODERATE. **—mit'i·ga·ble** (-gə-bəl) *adj.* **—mit'i·ga'tion** *n.* **—mit'i·ga'tive, mit'i·ga·to'ry** (-gə-tôr'ē, -tōr'ē) *adj.* **—mit'i·ga'tor** *n.*

mi·to·chon·dri·on (mī'tə-kŏn'drē-ən) *n., pl.* **-dri·a** (-drē-ə) [NLat. : Gk. *mitos*, thread + Gk. *khondrion*, dim. of *khondros*, grain.] *Biol.* A microscopic body occurring in the cells of nearly all living organisms and containing enzymes responsible for the conversion of food to usable energy. **—mi'to·chon'dri·al** (-drē-əl) *adj.*

mi·to·gen (mī'tə-jən) *n.* [MITO(SIS) + -GEN.] A mitosis-inducing agent. **—mi'to·gen'ic** (mī'tə-jĕn'ik, mit'ə-) *adj.* **—mi'to·ge·nic'i·ty** (-jə-nis'i-tē) *n.*

mi·to·my·cin (mī'tə-mī'sin) *n.* [MITO(SIS) + -MYCIN.] A complex of antibiotics produced by the bacterium *Streptomyces caespitosus* that is occas. used in cancer chemotherapy.

mi·to·sis (mī-tō'sis) *n., pl.* **-ses** (-sēz') [Gk. *mitos*, thread + -OSIS.] *Biol.* **1.** Sequential differentiation and segregation of replicated chromosomes in a cell nucleus that precedes complete cell division. **2.** The entire sequence of processes in cell division in which the diploid number of chromosomes is retained in both daughter cells. **—mi·tot'ic** (mī-tŏt'ik) *adj.* **—mi·tot'i·cal·ly** *adv.*

mi·tral (mī'trəl) *adj.* [Fr. < Lat. *mitra*, miter.] **1.** Relating to or resembling a miter. **2.** Relating to a mitral valve.

mitral valve *n.* The cardiac valve between the left auricle and the left ventricle regulating blood flow from the auricle to the ventricle.

mi·tre (mī'tər) *n.* & *v. Chiefly Brit. var. of* MITER.

mitt (mit) *n.* [Short for MITTEN.] **1.** A woman's glove extending over the hand but only partially covering the fingers. **2.** A mitten. **3.** A baseball glove, esp. a large leather padded glove used by catchers and first basemen. **4.** *Slang.* A hand : fist.

mit·ten (mit'n) *n.* [ME *mytan* < OFr. *mitaine*, prob. < Lat. *medietas*, half < *medius*, middle.] A covering for the hand that encases the thumb separately and the four fingers together.

mit·ti·mus (mit'ə-məs) *n., pl.* **-mus·es.** [Lat., we send (the first word of the writ).] *Law.* A writ instructing a jailer to hold a prisoner.

mitz·vah (mits'və) *n., pl.* **-voth** (-vōth', -vōt') *or* **-vahs.** [Heb. *miswāh.*] **1. a.** A commandment of the Jewish law. **b.** Fulfillment of such a commandment. **2.** A worthy deed.

mix (miks) *v.* **mixed, mix·ing, mix·es.** [Back-formation < obs. *mixte*, mixed < ME < OFr. < Lat. *mixtus*, p.part. of *miscēre*, to mix.] —*vt.* **1. a.** To combine or blend into one mass or mixture, rendering the constituent parts indistinguishable. **b.** To create or form by adding ingredients together. **c.** To add (an ingredient or element) to another. **2.** To combine or join <*mix* happiness with disappointment> **3.** To bring into social contact <*mix* young and old at a party> **4.** To crossbreed. —*vi.* **1. a.** To become mixed or blended together. **b.** To be capable of being blended together <Eggs *mix* with milk.> **2.** To associate socially or get along with others <*mix* with the crowd> **3.** To be crossbred. **—mix up. 1.** To confuse : confound. **2.** To involve <I got *mixed* up in a power struggle.> —*n.* **1.** An act of mixing. **2.** A mixture, esp. of ingredients packaged and sold commercially <muffin *mix*> **3.** A tape recording or a phonograph record produced by adjusting and combining sounds. **—mix it up.** *Slang.* To fight. **—mix'a·ble** *adj.*

mixed (mikst) *adj.* **1.** Blended together into a single unit or mass : INTERMINGLED. **2.** Composed of a variety of differing, sometimes conflicting entities <*mixed* reactions> **3.** Composed of people of different sexes, races, religions, or social classes.

mixed bag *n.* A collection of dissimilar things : ASSORTMENT.

mixed drink *n.* A drink of one or more kinds of liquor combined with other ingredients, usu. shaken or stirred before serving.

mixed grill *n.* A dish consisting of a variety of broiled meats and vegetables, typically including a lamb chop.

mixed marriage *n.* Marriage between persons of different races or religions.

mixed-me·di·a (mikst'mē'dē-ə) *adj.* Multimedia.

mixed metaphor *n.* A succession of metaphors that produce an incongruous and ludicrous effect, as *soaring ambitions that were stalled by a rock of resistance.*

mixed nerve *n.* A nerve having both sensory and motor fibers.

mixed number *n.* A number, as 7¼, equal to the sum of an integer and a fraction.

mixed-up (mikst'ŭp') *adj. Informal.* Muddled : confused.

mix·er (mik'sər) *n.* **1.** One that mixes. **2.** One who is sociable. **3.** An informal dance or party that gives members of a group a chance to become acquainted with each other. **4.** A device that blends or mixes substances or ingredients, esp. by mechanical agitation. **5.** A beverage, as soda water or ginger ale, for diluting alcoholic drinks.

mix·ol·o·gy (mik-sŏl'ə-jē) *n.* The study or skill of preparing mixed drinks. **—mix·ol'o·gist** *n.*

mixt (mikst) *v. Archaic. var. p.t. & p.p. of* MIX.

mix·ture (miks'chər) *n.* [Fr. < Lat. *mixtura* < *miscēre*, to mix.] **1.** Something made by mixing. **2.** Something composed of diverse elements <a *mixture* of rage and frustration> **3.** A fabric consisting of different kinds of thread or yarn. **4.** The act or process of mixing or state of being mixed. **5.** *Chem.* A composition of two or more substances not chemically bound to each other.

mix-up (miks'ŭp') *n.* **1.** Muddle : confusion. **2.** *Informal.* A fight.

Mi·zar (mī'zär') *n.* [Ar. *mi'zar*, Mizar, veil.] The star at the crook of the handle of the Big Dipper.

miz·zen *or* **miz·en** (miz'ən) *n.* [ME *meson* < OFr. *misaine*, prob. < Oltal. *mezzana* < *mezzano*, middle < Lat. *medianus* < *medius*, half.] *Naut.* **1.** A fore-and-aft sail set on the mizzenmast. **2.** A mizzenmast. **—miz'zen** *adj.*

miz·zen·mast *or* **miz·en·mast** (miz'ən-məst, -măst') *n. Naut.* **1.** The third mast aft on sailing ships carrying three or more masts. **2.** JIGGER MAST 1.

miz·zle¹ (miz'əl) *vi.* **-zled, -zling, -zles.** [ME *misellen.*] To rain in fine, mistlike droplets. **—miz'zle** *n.* **—miz'zly** *adv.*

miz·zle² (miz'əl) *vi.* **-zled, -zling, -zles.** [Orig. unknown.] *Chiefly Brit.* To make a sudden departure.

Mn *symbol for* MANGANESE.

mne·mon·ic (nī-mŏn'ik) *adj.* [Gk. *mnēmonikos*, of memory < *mnēmōn*, mindful < *mnasthai*, to remember.] Pertaining to, aiding, or intended to aid the memory. —*n.* A device, as a formula or rhyme, used as an aid in remembering. **—mne·mon'i·cal·ly** *adv.*

mne·mon·ics (nī-mŏn'iks) *n. (sing. in number).* A system to enhance or develop the memory.

Mne·mos·y·ne (nī-mŏs'ə-nē, -mŏz'-) *n.* [Lat. < Gk. *Mnēmosunē* < *mnasthai*, to remember.] *Gk. Myth.* The goddess of memory.

-mo *suff.* [< DUODECIMO.] —Used after numerals to indicate the number of leaves that results from folding a sheet of paper <twelvemo>

Mo *symbol for* MOLYBDENUM.

mo·a (mō'ə) *n.* [Maori.] Any of various large, long-necked, flightless birds of the order Dinorthiformes, native to New Zealand and extinct for more than a hundred years.

Mo·ab·ite (mō'ə-bīt') *n.* **1.** A descendant of Moab, the son of Lot in the Old Testament. **2.** An inhabitant of Moab. **—Mo'a·bite'** *adj.*

moan (mōn) *n.* [ME *mone*, complaint.] **1. a.** A low, drawn out, mournful sound, usu. indicating sorrow or pain. **b.** A sound similar to a moan. **2.** Lamentation. —*v.* **moaned, moan·ing, moans.** —*vi.* **1.** To utter a moan or make a sound like a moan. **2.** To complain, lament, or grieve. —*vt.* **1.** To bewail. **2.** To utter with a moan.

moat (mōt) *n.* [ME *mote* < OFr., mound.] A wide, deep ditch, usu. filled with water, around a medieval town, fortress, or castle for protection against assault. —*vt.* **moat·ed, moat·ing, moats.** To surround with or as if with a moat.

mob (mŏb) *n.* [Short for obs. *mobile* < Lat. *mobile (vulgus)*, ... (crowd).] **1.** A large disorderly crowd. **2.** The common people. **3.** *Informal.* An organized gang of criminals. —*vt.* **mobbed, mob·bing, mobs. 1.** To crowd around and jostle or annoy, esp. in anger or excessive enthusiasm <Dozens of shouting fans *mobbed* the actor.>

ured in steradians with respect to the area delimited on the unit sphere centered on that point by the locus of points of intersection of the sphere with the lines joining the point to the perimeter of the surface.

sol·i·dar·i·ty (sŏl'ĭ-dăr'ĭ-tē) *n.* A union of interests, sympathies, or purposes among the members of a group : FELLOWSHIP.

solid geometry *n.* The geometry of three-dimensional figures and surfaces.

sol·i·di (sŏl'ĭ-dī') *n. pl. of* SOLIDUS.

so·lid·i·fy (sə-lĭd'ə-fī') *vt. & vi.* **-fied, -fy·ing, -fies.** 1. To make or become solid or united. **—so·lid'i·fi·ca'tion** *n.*

so·lid·i·ty (sə-lĭd'ĭ-tē) *n.* 1. The state or property of being solid. 2. Something solid.

solid of revolution *n.* A volume generated by the rotation of a plane figure about an axis in its plane.

solid propellant *n.* A rocket propellant in solid form, combining both fuel and oxidizer in the form of a compact, cohesive grain.

solid solution *n. Chem.* A homogeneous crystalline structure in which one or more types of atoms or molecules may be partly substituted for the original atoms and molecules without changing the structure.

sol·id-state (sŏl'ĭd-stāt') *adj.* 1. Relating to or characteristic of the physical properties of solid materials, esp. to the electromagnetic, thermodynamic, and structural properties of crystalline solids. 2. Based on or composed chiefly or exclusively of semiconducting materials, components, and related devices.

sol·i·dus (sŏl'ĭ-dəs) *n., pl.* **-di** (-dī') [ME < Lat. < *solidus*, solid.] 1. An ancient Roman coin used until the fall of the Byzantine Empire. 2. A virgule.

so·lil·o·quize (sə-lĭl'ə-kwīz') *vi. & vt.* **-quized, -quis·ing, -quiz·es.** To utter or put into a soliloquy. **—so·lil'o·quist, so·lil'o·quiz'er** *n.*

so·lil·o·quy (sə-lĭl'ə-kwē) *n., pl.* **-quies.** [LLat. : Lat. *solus*, alone + Lat. *loqui*, to speak.] 1. Literary or dramatic discourse in which a character talks to himself or herself or reveals his or her thoughts in the form of a monologue without addressing a listener. 2. The act of speaking to oneself.

sol·ip·sism (sŏl'ĭp-sĭz'əm, sō'lĭp-) *n.* [Lat. *solus*, alone + Lat. *ipse*, self + -ISM.] *Philos.* 1. The theory that the self is the only thing that can be known and verified. 2. The theory or view that the self is the only reality. **—sol'ip·sist** *n.* **—sol'ip·sis'tic** *adj.*

sol·i·taire (sŏl'ĭ-târ') *n.* [Fr. < OFr., solitary < Lat. *solitarius* < *solus*, alone.] 1. A gem, as a diamond, set alone. 2. Any of a number of card games played by one person.

sol·i·tary (sŏl'ĭ-tĕr'ē) *adj.* [ME < Lat. *solitarius* < *solus*, alone.] 1. Existing, living, or acting without others <a *solitary* passenger> 2. Occurring, carried out, or made alone <a *solitary* evening> 3. Remote : secluded <a *solitary* mountain retreat> 4. Standing alone : SOLE <a *solitary* pine standing on the hill> —*n., pl.* **-ies.** 1. One who lives alone : RECLUSE. 2. *Informal.* Solitary confinement. **—sol'i·tar'i·ly** (-tăr'ə-lē) *adv.* **—sol'i·tar'i·ness** *n.*

solitary confinement *n.* Confinement of a prisoner in a cell in which he or she is isolated from all others.

sol·i·tude (sŏl'ĭ-tōōd', -työōd') *n.* [ME < OFr. < Lat. *solitudo* < *solus*, alone.] 1. The quality or state of being alone or remote from others. 2. A lonely or secluded place.

☆ **syns:** SOLITUDE, ISOLATION *n. core meaning* : the quality or state of being alone <preferred *solitude* to crowded streets>

sol·i·tud·i·nar·i·an (sŏl'ĭ-tōōd'ə-târ'ē-ən, -työōd'-) *n.* [Lat. *solitudo, solitudin-*, solitude + -ARIAN.] A recluse.

sol·ler·et (sŏl'ə-rĕt') *n.* [OFr., dim. of *soller*, shoe.] A steel shoe of overlapping plates, worn as part of medieval armor.

sol·mi·za·tion (sŏl'mĭ-zā'shən) *n.* [Fr. *solmisation* < *solmiser*, to sol-fa.] *Mus.* The act or a system of using syllables, as *do, re,* and *mi,* to represent the tones of the scale.

so·lo (sō'lō) *n., pl.* **-los.** [Ital. < Lat. *solus*, alone.] 1. An accompanied or unaccompanied musical composition or passage for a single voice or instrument. 2. Something performed by one person. 3. A card game in which one player singly opposes others. —*adj.* 1. Composed, arranged for, or performed by a single voice or instrument. 2. Made or carried out by one person. —*adv.* Unaccompanied : alone. —*vi.* **-loed, -lo·ing, -los.** To perform alone, esp. to fly an aircraft without a companion or instructor.

so·lo·ist (sō'lō-ĭst) *n.* A solo performer.

Solomon's seal *n.* [After *Solomon*, 10th-cent. king of Israel.] 1. A six-pointed star or hexagram held to possess mystical powers. 2. Any of several plants of the genus *Polygonatum*, with paired, drooping, greenish or yellowish flowers.

So·lon (sō'lən, -lŏn') *n.* [After *Solon*, Athenian statesman of the 7th to 6th cent. B.C.] 1. A wise lawgiver. 2. A member of a legislature.

so long *interj. Informal.* —Used to express farewell.

sol·stice (sŏl'stĭs, sōl'-, sōl'-) *n.* [ME < OFr. < Lat. *solstitium* : *sol*, sun + *sistere*, to stand.] 1. *Astron.* Either of two times of the year when the sun has no apparent northward or southward motion, at the most northern or most southern point of the ecliptic; the summer solstice, when the sun is in the zenith at the tropic of Cancer, occurs about Jun. 22, and the winter solstice, when it is over the tropic of Capricorn, occurs about Dec. 22. 2. A highest point : ZENITH. **—sol·sti'tial** (-stĭsh'əl) *adj.*

sol·u·bi·lize (sŏl'yə-bə-līz') *vt.* **-lized, -liz·ing, -liz·es.** To make (substances such as fats and lipids, which are not appreciably soluble under standard conditions) soluble in water by the action of a detergent or similar agent.

sol·u·ble (sŏl'yə-bəl) *adj.* [ME < OFr. < LLat. *solubilis* < *solvere*, to loosen.] 1. Capable of being dissolved. 2. Capable of being explained or solved. **—sol'u·bil'i·ty** (-bĭl'ĭ-tē) *n.* **—sol'u·ble·ness** *n.* **—sol'u·bly** *adv.*

soluble glass *n.* Sodium silicate.

so·lum (sō'ləm) *n., pl.* **-la** (-lə) or **-lums.** [NLat. < Lat., foundation.] The surface layers of a soil profile in which topsoil formation occurs.

so·lus (sō'ləs) *adj. & adv.* [Lat., alone.] By oneself : ALONE. —Used as a stage direction.

sol·ute (sŏl'yōōt') *n.* [< Lat. *solutus*, p.part. of *solvere*, to loosen.] A substance dissolved in another substance, usu. the component of a solution present in the lesser amount. **—sol'ute'** *adj.*

so·lu·tion (sə-lōō'shən) *n.* [ME < OFr. < Lat. *solutio* < *solutus*, p.part. of *solvere*, to loosen.] 1. a. A spontaneously forming homogeneous mixture of two or more substances, retaining its constitution in subdivision to molecular volumes, displaying no settling, and having various possible proportions of the constituents, which may be solids, liquids, gases, or intercombinations. b. Formation of such a mixture. c. The state of being dissolved. 2. a. The method or process of solving a problem. b. The answer to or disposition of a problem. 3. *Law.* Payment or satisfaction of a claim or debt. 4. The act of separating or breaking up : DISSOLUTION.

So·lu·tre·an *also* **So·lu·tri·an** (sə-lōō'trē-ən) *adj.* [After *Solutré*, France.] Of or relating to the Old World Upper Paleolithic culture that succeeded the Aurignacian and was marked by improved flint implements and stylized symbolic forms of art.

sol·va·tion (sŏl-vā'shən, sōl-) *n.* [SOLV(ENT) + -ATION.] Any of a class of chemical reactions, such as formation of hydrated copper sulfate in aqueous solution, in which solute and solvent molecules combine with relatively weak covalent bonds.

Sol·vay process (sŏl'vā') *n.* [After Ernest *Solvay* (1838–1932).] A process used to produce large quantities of sodium bicarbonate from salt, ammonia, carbon dioxide, and limestone.

solve (sŏlv, sōlv) *vt.* **solved, solv·ing, solves.** [ME *solven*, to loosen < Lat. *solvere.*] 1. To find a solution to. 2. To work out a correct solution to (a problem). **—solv'a·bil'i·ty, solv'a·ble·ness** *n.* **—solv'a·ble** *adj.* **—solv'er** *n.*

sol·vent (sŏl'vənt, sōl'-) *adj.* [Lat. *solvens, solvent-*, pr.part. of *solvere*, to loosen.] 1. Able to meet one's financial obligations. 2. Capable of dissolving another substance. —*n.* 1. *Chem.* a. The component of a solution that is present in excess or that undergoes no change of state. b. A liquid capable of dissolving another substance. 2. Something that solves. **—sol'ven·cy** *n.*

sol·vol·y·sis (sŏl-vŏl'ĭ-sĭs, sōl-) *n.* [SOLV(ENT) + -LYSIS.] Any of a class of ionic chemical reactions, as hydrolysis, in which solute and solvent react and alter the acidity or relative ionic concentrations of the solution. **—sol'vo·lyt'ic** (-və-lĭt'ĭk) *adj.*

so·ma (sō'mə) *n., pl.* **-ma·ta** (-mə-tə) *or* **-mas.** [NLat. < Gk. *sōma*, body.] *Biol.* The physical entity of an organism, exclusive of the germ cells.

So·ma·li (sō-mä'lē) *n., pl.* **Somali** *or* **-lis.** 1. A member of one of a group of Hamitic tribes of Somaliland. 2. The Cushitic language of the Somali.

so many *adj.* 1. Forming an unspecified number <issued *so many* regulations each year> 2. Forming a pack or group <fought like *so many* tigers>

so·ma·ta (sō'mə-tə) *n. var. pl. of* SOMA.

so·mat·ic (sō-măt'ĭk) *adj.* [Gk. *sōmatikos* < *sōma*, body.] 1. Of or relating to the body : PHYSICAL. 2. Of or relating to the wall of the body cavity. 3. Of or relating to somatoplasm. **—so·mat'i·cal·ly** *adv.*

somatic cell *n.* A bodily cell other than a germ cell.

somato- *pref.* [< Gk. *sōma, sōmat-*, body.] 1. Body <*somatology*> 2. Soma <*somatoplasm*>

so·mat·o·gen·ic (sō-măt'ə-jĕn'ĭk) *also* **so·mat·o·ge·net·ic** (-jə-nĕt'ĭk) *adj.* Arising within the body in response to environment.

so·ma·tol·o·gy (sō'mə-tŏl'ə-jē) *n.* 1. Physiological and anatomical study of the body. 2. Physical anthropology. **—so'ma·to·log'i·cal** (sō'mə-tə-lŏj'ĭ-kəl, sō-măt'ə-) *adj.*

so·mat·o·plasm (sō-măt'ə-plăz'əm) *n.* 1. The entirety of specialized protoplasm, other than germ plasm, that constitutes the body. 2. The protoplasm of a somatic cell.

so·mat·o·pleure (sō-măt'ə-plōōr') *n.* [NLat. *somatopleura* : SOMATO- + Gk. *pleura*, side.] A complex sheet of embryonic cells in certain vertebrates, formed by association of part of the mesoderm with the ectoderm and developing as the internal body wall. **—so·mat'o·pleu'ric** (-plōōr'ĭk) *adj.*

so·mat·o·type (sō-măt'ə-tīp') *n.* The morphological type of a human body : PHYSIQUE. **—so·mat'o·typ'ic** (-tĭp'ĭk) *adj.*

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1 2 3 4 5 6 7 8 9

THROMBIN IS HOMOLOGOUS TO TRYPSIN

The specificity of thrombin for arginine–glycine bonds suggests that thrombin might resemble trypsin. Indeed it does, as shown by amino acid sequence studies. Thrombin has a molecular weight of 33,700 and consists of two chains. The A chain of 49 residues exhibits no detectable homology to the pancreatic enzymes. The B chain, however, is quite similar in sequence to trypsin, chymotrypsin, and elastase. The sequence around its active-site serine is Gly-Asp-Ser-Gly-Gly-Pro, the same as that in the pancreatic serine proteases. Moreover, thrombin also contains a charge relay network. Its three-dimensional structure is not yet known, but one important feature of its specificity site is already apparent. Thrombin, like trypsin, contains an aspartate residue at the bottom of its substrate binding cleft. This negatively charged group undoubtedly forms an electrostatic bond with the positively charged arginine side chain.

Thrombin is much more specific than trypsin. Thrombin cleaves certain arginine–glycine bonds, whereas trypsin cleaves most peptide bonds following arginine or lysine residues. Thrombin, like the pancreatic serine proteases, is synthesized as a zymogen called *prothrombin,* which has a molecular weight of about 70,000. The activation mechanism appears to be more complex than in the homologous pancreatic enzymes. However, at least one feature of the activation process is common to these enzymes. An ion pair like the one formed between the positively charged amino group of isoleucine 16 and the negatively charged aspartate 194 in chymotrypsin also occurs in thrombin.

Similarities in amino acid sequence indicate that *thrombin is evolutionarily related to the pancreatic serine proteases.* This is reinforced by the occurrence of a charge relay system and the likelihood of a similar activation mechanism. It is of interest to note that thrombin is formed in the liver, which has a common embryological origin with the pancreas.

PROTHROMBIN IS ACTIVATED BY FACTOR X_a

The cascade nature of the clotting process is evident in Figure 8-28. Prothrombin is activated by a proteolytic enzyme which is called Factor X_a. Clotting factors are assigned a Roman numeral for ease

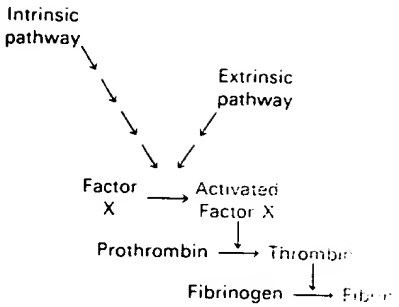

Figure 8-28
Final steps in the formation of a fibrin clot. These three reactions constitute the common pathway. Factor X is activated by products of the intrinsic and extrinsic pathways.